

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7:

C12N 15/52, 15/54, 15/62, 9/10, C12P 17/18, 19/32, C07D 498/18 // (C07D 498/18, 311:00, 273:00, 211:00)

(11) International Publication Number:

WO 00/20601

(43) International Publication Date:

13 April 2000 (13.04.00)

(21) International Application Number:

PCT US99 22886

A2

(22) International Filing Date:

1 October 1999 (01.10.99)

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(30) Priority Data:

60/102,748 2 October 1998 (02.10.98) US 60/123,810 11 March 1999 (11.03.99) US 60/139,650 17 June 1999 (17.06.99) US (81) Designated States: AL, AM, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, IL, IS, JP, KG, KP, KR, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

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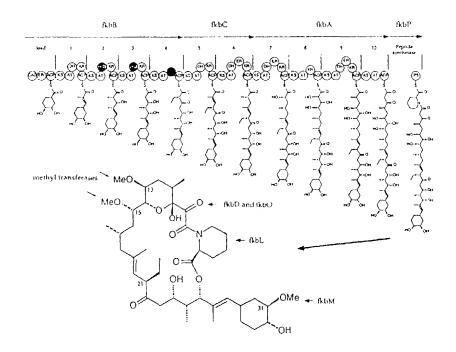
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Published

Without international search report and to be republished upon receipt of that report.

(54) Title: POLYKETIDE SYNTHASÉ ENZYMES AND RECOMBINANT DNA CONSTRUCTS THEREFOR



(57) Abstract

Host cells comprising recombinant vectors encoding the FK-520 polyketide synthase and FK-520 modification enzymes can be used to produce the FK-520 polyketide. Recombinant DNA constructs comprising one or more FK-520 polyketide synthase domains, modules, open reading frames, and variants thereof can be used to produce recombinant polyketide synthases and a variety of different polyketides with application as pharmaceutical and veterinary products.

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POLYKETIDE SYNTHASE ENZYMES AND RECOMBINANT DNA CONSTRUCTS THEREFOR

Field of the Invention

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The present invention relates to polyketides and the polyketide synthase (PKS) enzymes that produce them. The invention also relates generally to genes encoding PKS enzymes and to recombinant host cells containing such genes and in which expression of such genes leads to the production of polyketides. The present invention also relates to compounds useful as medicaments having immunosuppressive and/or neurotrophic activity. Thus, the invention relates to the fiel of chemistry, molecular biology, and agricultural, medical, and veterinary technology.

Background of the Invention

Polyketides are a class of compounds synthesized from 2-carbon units through a series of condensations and subsequent modifications. Polyketides occur in many types of organisms, including fungi and mycelial bacteria, in particular, the actinomycetes. Polyketides are biologically active molecules with a wide variety of structures, and the class encompasses numerous compounds with diverse activities. Tetracycline, erythromycin, epothilone, FK-506, FK-520, narbomycin, picromycin, rapamycin, spinocyn, and tylosin are examples of polyketides. Given the difficulty in producing polyketide compounds by traditional chemical methodology, and the typically low production of polyketides in wild-type cells, there has been considerable interest in finding improved or alternate means to produce polyketide compounds.

This interest has resulted in the cloning, analysis, and manipulation by recombinant DNA technology of genes that encode PKS enzymes. The resulting technology allows one to manipulate a known PKS gene cluster either to produce the polyketide synthesized by that PKS at higher levels than occur in nature or in hosts that otherwise do not produce the polyketide. The technology also allows one to produce molecules that are structurally related to, but distinct from, the polyketides produced from known PKS gene clusters. See, e.g., PCT publication Nos. WO 93/13663; 95/08548; 96/40968; 97/02358; 98/27203; and 98/49315; United States Patent Nos. 4,874,748; 5,063,155; 5,098,837; 5,149,639; 5,672,491; 5,712,146; 5,830,750; and 5,843,718; and Fu et al., 1994, Biochemistry 33: 9321-9326; McDaniel et al., 1993, Science 262: 1546-1550; and Rohr, 1995, Angew. Chem. Int. Ed. Engl. 34(8): 881-888, each of which is incorporated herein by reference.

Polyketides are synthesized in nature by PKS enzymes. These enzymes, which are complexes of multiple large proteins, are similar to the synthases that catalyze condensation of 2-carbon units in the biosynthesis of fatty acids. PKSs catalyze the biosynthesis of polyketides through repeated, decarboxylative Claisen condensations between acylthioester building blocks. The building blocks used to form complex polyketides are typically acylthioesters, such as acetyl, butyryl, propionyl, malonyl, hydroxymalonyl, methylmalonyl, and ethylmalonyl CoA. Other building blocks include amino acid like acylthioesters. PKS enzymes that incorporate such building blocks include an activity that functions as an amino acid ligase (an AMP ligase) or as a non-ribosomal patide synthesize (NRPS). Two major types of PKS enzymes are known; these differ in their composition and mode of synthesis of the polyketide synthesized. These two major types of PKS enzymes are commonly referred to as Type I or "modular" and Type II "iterative" PKS enzymes.

In the Type I or modular PKS enzyme group, a set of separate catalytic active sites (each active site is termed a "domain", and a set thereof is termed a "module") exists for each cycle of carbon chain elongation and modification in the polyketide synthesis pathway. The typical modular PKS is composed of several large polypeptides, which can be segregated from amino to carboxy termini into a loading module, multiple extender modules, and a releasing (or thioesterase) domain. The PKS enzyme known as 6-deoxyerythronolide B synthase (DEBS) is a Type I PKS. In DEBS, there is a loading module, six extender modules, and a thioesterase (TE) domain. The loading module, six extender modules, and TE of DEBS are present on three separate proteins (designated DEBS-1, DEBS-2, and DEBS-3, with two extender modules per protein). Each of the DEBS polypeptides is encoded by a separate open reading frame (ORF) or gene; these genes are known as *eryAI*, *eryAII*, and *eryAIII*. See Caffrey *et al.*, 1992, *FEBS Letters* 304: 205, and U.S. Patent No. 5,824,513, each of which is incorporated herein by reference.

Generally, the loading module is responsible for binding the first building block used to synthesize the polyketide and transferring it to the first extender module. The loading module of DEBS consists of an acyltransferase (AT) domain and an acyl carrier protein (ACP) domain. Another type of loading module utilizes an inactivated ketosynthase (KS) domain and AT and ACP domains. This inactivated KS is in some instances called KS^Q, where the superscript letter is the abbreviation for the amino acid, glutainine, that is present instead of the active site cysteine required for ketosynthase activity. In other PKS enzymes, including the FK-506 PKS, the loading module

incorporates an unusual starter unit and is composed of a CoA ligase like activity domain. In any event, the loading module recognizes a particular acyl-CoA (usually acetyl or propionyl but sometimes butyryl or other acyl-CoA) and transfers it as a thiol ester to the ACP of the loading module.

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The AT on each of the extender modules recognizes a particular extender-CoA (malonyl or alpha-substituted malonyl, i.e., methylmalonyl, ethylmalonyl, and 2-hydroxymalonyl) and transfers it to the ACP of that extender module to form a thioester. Each extender module is responsible for accepting a compound from a prior module, binding a building block, attaching the building block to the compound from the prior module, optionally performing one or more additional range one, and transferring the resulting compound to the next module.

Each extender module of a modular PKS contains a KS. AT. ACP, and zero, one, two, or three domains that modify the beta-carbon of the growing polyketide chain. A typical (non-loading) minimal Type I PKS extender module is exemplified by extender module three of DEBS, which contains a KS domain, an AT domain, and an ACP domain. These three domains are sufficient to activate a 2-carbon extender unit and attach it to the growing polyketide molecule. The next extender module, in turn, is responsible for attaching the next building block and transferring the growing compound to the next extender module until synthesis is complete.

Once the PKS is primed with acyl- and malonyl-ACPs, the acyl group of the loading module is transferred to form a thiol ester (trans-esterification) at the KS of the first extender module; at this stage, extender module one possesses an acyl-KS and a malonyl (or substituted malonyl) ACP. The acyl group derived from the loading module is then covalently attached to the alpha-carbon of the malonyl group to form a carbon-carbon bond, driven by concomitant decarboxylation, and generating a new acyl-ACP that has a backbone two carbons longer than the loading building block (elongation or extension).

The polyketide chain, growing by two carbons each extender module, is sequentially passed as covalently bound thiol esters from extender module to extender module, in an assembly line-like process. The carbon chain produced by this process alone would possess a ketone at every other carbon atom, producing a polyketone, from which the name polyketide arises. Most commonly, however, additional enzymatic activities modify the beta keto group of each two carbon unit just after it has been added to the growing polyketide chain but before it is transferred to the next module.

Thus, in addition to the minimal module containing KS, AT, and ACP domains necessary to form the carbon-carbon bond, and as noted above, other domains that modify the beta-carbonyl moiety can be present. Thus, modules may contain a ketoreductase (KR) domain that reduces the keto group to an alcohol. Modules may also contain a KR domain plus a dehydratase (DH) domain that dehydrates the alcohol to a double bond. Modules may also contain a KR domain, a DH domain, and an enoylreductase (ER) domain that converts the double bond product to a saturated single bond using the beta carbon as a methylene function. An extender module can also contain other enzymatic activity, such as, for example, a methylase or dimethylase activity.

After traversing the final extender module, the polyketide encounters a releasing domain that cleaves the polyketide from the PKS and typically cyclizes the polyketide. For example, final synthesis of 6-dEB is regulated by a TE domain located at the end of extender module six. In the synthesis of 6-dEB, the TE domain catalyzes cyclization of the macrolide ring by formation of an ester linkage. In FK-506, FK-520, rapamycin, and similar polyketides, the TE activity is replaced by a RapP (for rapamycin) or RapP like activity that makes a linkage incorporating a pipecolate acid residue. The enzymatic activity that catalyzes this incorporation for the rapamycin enzyme is known as RapP, encoded by the *rapP* gene. The polyketide can be modified further by tailoring enzymes; these enzymes add carbohydrate groups or methyl groups, or make other modifications. i.e., oxidation or reduction, on the polyketide core molecule. For example, 6-dEB is hydroxylated at C-6 and C-12 and glycosylated at C-3 and C-5 in the synthesis of ervthromycin A.

In Type I PKS polypeptides, the order of catalytic domains is conserved. When all beta-keto processing domains are present in a module, the order of domains in that module from N-to-C-terminus is always KS, AT, DH, ER, KR, and ACP. Some or all of the beta-keto processing domains may be missing in particular modules, but the order of the domains present in a module remains the same. The order of domains within modules is believed to be important for proper folding of the PKS polypetides into an active complex. Importantly, there is considerable flexibility in PKS enzymes, which allows for the genetic engineering of novel catalytic complexes. The engineering of these enzymes is achieved by modifying, adding, or deleting domains, or replacing them with those taken from other Type I PKS enzymes. It is also achieved by deleting, replacing, or adding entire modules with those taken from other sources. A genetically engineered

PKS complex should of course have the ability to catalyze the synthesis of the product predicted from the genetic alterations made.

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Alignments of the many available amino acid sequences for Type I PKS enzymes has approximately defined the boundaries of the various catalytic domains. Sequence alignments also have revealed linker regions between the catalytic domains and at the Nand C-termini of individual polypeptides. The sequences of these linker regions are less well conserved than are those for the catalytic domains, which is in part how linker regions are identified. Linker regions can be important for proper association between domains and between the individual polypeptides that comprise the PKS complex. One can thus view the linkers and domains together as creating a scaffold on which the domains and modules are positioned in the correct orientation to be active. This organization and positioning, if retained, permits PKS domains of different or identical substrate specificities to be substituted (usually at the DNA level) between PKS enzymes by various available methodologies. In selecting the boundaries of, for example, an AT replacement, one can thus make the replacement so as to retain the linkers of the recipient PKS or to replace them with the linkers of the donor PKS AT domain, or, preferably, make both constructs to ensure that the correct linker regions between the KS and AT domains have been included in at least one of the engineered enzymes. Thus, there is considerable flexibility in the design of new PKS enzymes with the result that known polyketides can be produced more effectively, and novel polyketides useful as pharmaceuticals or for other purposes can be made.

By appropriate application of recombinant DNA technology, a wide variety of polyketides can be prepared in a variety of different host cells provided one has access to nucleic acid compounds that encode PKS proteins and polyketide modification enzymes. The present invention helps meet the need for such nucleic acid compounds by providing recombinant vectors that encode the FK-520 PKS enzyme and various FK-520 modification enzymes. Moreover, while the FK-506 and FK-520 polyketides have many useful activities, there remains a need for compounds with similar useful activities but with better pharmacokinetic profile and metabolism and fewer side-effects. The present invention helps meet the need for such compounds as well.

Summary of the Invention

In one embodiment, the present invention provides recombinant DNA vectors that encode all or part of the FK-520 PKS enzyme. Illustrative vectors of the invention include cosmid pKOS034-120, pKOS034-124, pKOS065-C31, pKOS065-C3,

pKOS065-M27, and pKOS065-M21. The invention also provides nucleic acid compounds that encode the various domains of the FK-520 PKS, i.e., the KS, AT, ACP, KR, DH, and ER domains. These compounds can be readily used, alone or in combination with nucleic acids encoding other FK-520 or non-FK-520 PKS domains, as intermediates in the construction of recombinant vectors that encode all or part of PKS enzymes that make novel polyketides.

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The invention also provides isolated nucleic acids that encode all or part of one or more modules of the FK-520 PKS, each module comprising a ketosynthase activity, an acyl transferase activity, and an acyl carrier protein activity. The invention provides an isolated nucleic acid that encodes one or more open reading frames of FK-520 PKS genes, said open reading frames comprising coding sequences for a CoA ligase activity, an NRPS activity, or two or more extender modules. The invention also provides recombinant expression vectors containing these nucleic acids.

In another embodiment, the invention provides isolated nucleic acids that encode all or a part of a PKS that contains at least one module in which at least one of the domains in the module is a domain from a non-FK-520 PKS and at least one domain is from the FK-520 PKS. The non-FK-520 PKS domain or module originates from the rapamycin PKS, the FK-506 PKS, DEBS, or another PKS. The invention also provides recombinant expression vectors containing these nucleic acids.

In another embodiment, the invention provides a method of preparing a polyketide, said method comprising transforming a host cell with a recombinant DNA vector that encodes at least one module of a PKS, said module comprising at least one FK-520 PKS domain, and culturing said host cell under conditions such that said PKS is produced and catalyzes synthesis of said polyketide. In one aspect, the method is practiced with a *Streptomyces* host cell. In another aspect, the polyketide produced is FK-520. In another aspect, the polyketide produced is a polyketide related in structure to FK-520. In another aspect, the polyketide produced is a polyketide related in structure to FK-506 or rapamycin.

In another embodiment, the invention provides a set of genes in recombinant form sufficient for the synthesis of ethylmalonyl CoA in a heterologous host cell. These genes and the methods of the invention enable one to create recombinant host cells with the ability to produce polyketides or other compounds that require ethylmalonyl CoA for biosynthesis. The invention also provides recombinant nucleic acids that encode AT domains specific for ethylmalonyl CoA. Thus, the compounds of the invention can be

used to produce polyketides requiring ethylmalonyl CoA in host cells that otherwise are unable to produce such polyketides.

In another embodiment, the invention provides a set of genes in recombinant form sufficient for the synthesis of 2-hydroxymalonyl CoA and 2-methoxymalonyl CoA in a heterologous host cell. These genes and the methods of the invention enable one to create recombinant host cells with the ability to produce polyketides or other compounds that require 2-hydroxymalonyl CoA for biosynthesis. The invention also provides recombinant nucleic acids that encode AT domains specific for 2-hydroxymalonyl CoA and 2-methoxymalonyl CoA. Thus, the compounds of the invention can be used to produce polyketides requiring 2-hydroxymalonyl CoA or 2-methoxymalonyl CoA in host cells that are otherwise unable to produce such polyketides.

In another embodiment, the invention provides a compound related in structure to FK-520 or FK-506 that is useful in the treatment of a medical condition. These compounds include compounds in which the C-13 methoxy group is replaced by a moiety selected from the group consisting of hydrogen, methyl, and ethyl moieties. Such compounds are less susceptible to the main *in vivo* pathway of degradation for FK-520 and FK-506 and related compounds and thus exhibit an improved pharmacokinetic profile. The compounds of the invention also include compounds in which the C-15 methoxy group is replaced by a moiety selected from the group consisting of hydrogen, methyl, and ethyl moieties. The compounds of the invention also include the above compounds further modified by chemical methodology to produce derivatives such as, but not limited to, the C-18 hydroxyl derivatives, which have potent neurotrophin but not immunosuppression activities.

Thus, the invention provides polyketides having the structure:

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wherein, R₁ is hydrogen, methyl, ethyl, or allyl; R₂ is hydrogen or hydroxyl, provided that when R₂ is hydrogen, there is a double bond between C-20 and C-19; R₃ is hydrogen

or hydroxyl; R₄ is methoxyl, hydrogen, methyl, or ethyl; and R₅ is methoxyl, hydrogen, methyl, or ethyl; but not including FK-506, FK-520, 18-hydroxy-FK-520, and 18-hydroxy-FK-506. The invention provides these compounds in purified form and in pharmaceutical compositions.

In another embodiment, the invention provides a method for treating a medical condition by administering a pharmaceutically efficacious dose of a compound of the invention. The compounds of the invention may be administered to achieve immunosuppression or to stimulate nerve growth and regeneration.

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These and other embodiments and aspects of the invention will be more fully understood after consideration of the attached Drawings and the above below, together with the detailed description, examples, and claims that follow.

Brief Description of the Drawings

Figure 1 shows a diagram of the FK-520 biosynthetic gene cluster. The top line provides a scale in kilobase pairs (kb). The second line shows a restriction map with selected restriction enzyme recognition sequences indicated. K is *KpnI*; X is *XhoI*, S is *SacI*; P is *PstI*; and E is *EcoRI*. The third line indicates the position of FK-520 PKS and related genes. Genes are abbreviated with a one letter designation, i.e., C is *fkbC*. Immediately under the third line are numbered segments showing where the loading module (L) and ten different extender modules (numbered 1 - 10) are encoded on the various genes shown. At the bottom of the Figure, the DNA inserts of various cosmids of the invention (i.e., 34-124 is cosmid pKOS034-124) are shown in alignment with the FK-520 biosynthetic gene cluster.

Figure 2 shows the loading module (load), the ten extender modules, and the peptide synthetase domain of the FK-520 PKS, together with, on the top line, the genes that encode the various domains and modules. Also shown are the various intermediates in FK-520 biosynthesis, as well as the structure of FK-520, with carbons 13, 15, 21, and 31 numbered. The various domains of each module and subdomains of the loading module are also shown. The darkened circles showing the DH domains in modules 2, 3, and 4 indicate that the dehydratase domain is not functional as a dehydratase; this domain may affect the stereochemistry at the corresponding position in the polyketide. The substituents on the FK-520 structure that result from the action of non-PKS enzymes are also indicated by arrows, together with the types of enzymes or the genes that code for the enzymes that mediate the action. Although the methyltransferase is shown acting at the C-13 and C-15 hydroxyl groups after release of the polyketide from the PKS, the

methyltransferase may act on the 2-hydroxymalonyl substrate prior to or contemporaneously with its incorporation during polyketide synthesis.

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Figure 3 shows a close-up view of the left end of the FK-520 gene cluster, which contains at least ten additional genes. The ethyl side chain on carbon 21 of FK-520 (Figure 2) is derived from an ethylmalonyl CoA extender unit that is incorporated by an ethylmalonyl specific AT domain in extender module 4 of the PKS. At least four of the genes in this region code for enzymes involved in ethylmalonyl biosynthesis. The polyhydroxybutyrate depolymerase is involved in maintaining hydroxybutyryl-CoA pools during FK-520 production. Polyhydroxybutyrate accumulates during vegetative growth and disappears during stationary phase in other *Streptomyces* (Ranade and Vining, 1993, *Can. J. Microbiol. 39*:377). Open reading frames with unknown function are indicated with a question mark.

Figure 4 shows a biosynthetic pathway for the biosynthesis of ethylmalonyl CoA from acetoacetyl CoA consistent with the function assigned to four of the genes in the FK-520 gene cluster shown in Figure 3.

Figure 5 shows a close-up view of the right-end of the FK-520 PKS gene cluster (and of the sequences on cosmid pKOS065-C31). The genes shown include *fkbD*, *fkbM* (a methyl transferase that methylates the hydroxyl group on C-31 of FK-520), *fkbN* (a homolog of a gene described as a regulator of cholesterol oxidase and that is believed to be a transcriptional activator), *fkbQ* (a type II thioesterase, which can increase polyketide production levels), and *fkbS* (a crotonyl-CoA reductase involved in the biosynthesis of ethylmalonyl CoA).

Figure 6 shows the proposed degradative pathway for tacrolimus (FK-506) metabolism.

Figure 7 shows a schematic process for the construction of recombinant PKS genes of the invention that encode PKS enzymes that produce 13-desmethoxy FK-506 and FK-520 polyketides of the invention, as described in Example 4, below.

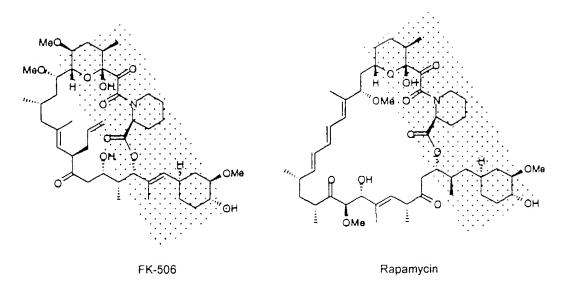
Figure 8, in Parts A and B, shows certain compounds of the invention preferred for dermal application in Part A and a synthetic route for making those compounds in Part B.

Detailed Description of the Invention

Given the valuable pharmaceutical properties of polyketides, there is a need for methods and reagents for producing large quantities of polyketides, as well as for producing related compounds not found in nature. The present invention provides such

methods and reagents, with particular application to methods and reagents for producing the polyketides known as FK-520, also known as ascomycin or L-683,590 (see Holt *et al.*, 1993, *JACS 115*:9925), and FK-506, also known as tacrolimus. Tacrolimus is a macrolide immunosuppressant used to prevent or treat rejection of transplanted heart, kidney, liver, lung, pancreas, and small bowel allografts. The drug is also useful for the prevention and treatment of graft-versus-host disease in patients receiving bone marrow transplants, and for the treatment of severe, refractory uveitis. There have been additional reports of the unapproved use of tacrolimus for other conditions, including alopecia universalis, autoimmune chronic active hepatitis, inflammatory bowel disease, multiple sclerosis, primary biliary cirrhosis, and scleroderma. The invention provides methods and reagents for making novel polyketides related in structure to FK-520 and FK-506, and structurally related polyketides such as rapamycin.

The FK-506 and rapamycin polyketides are potent immunosuppressants, with chemical structures shown below.



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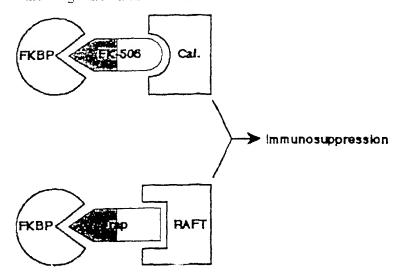
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FK-520 differs from FK-506 in that it lacks the allyl group at C-21 of FK-506, having instead an ethyl group at that position, and has similar activity to FK-506, albeit reduced immunosuppressive activity.

These compounds act through initial formation of an intermediate complex with protein "immunophilins" known as FKBPs (FK-506 binding proteins), including FKBP-12. Immunophilins are a class of cytosolic proteins that form complexes with molecules such as FK-506, FK-520, and rapamycin that in turn serve as ligands for other cellular targets involved in signal transduction. Binding of FK-506, FK-520, and rapamycin to FKBP occurs through the structurally similar segments of the polyketide molecules, known as the "FKBP-binding domain" (as generally but not precisely indicated by the

stippled regions in the structures above). The FK-506-FKBP complex then binds calcineurin, while the rapamycin-FKBP complex binds to a protein known as RAFT-1. Binding of the FKBP-polyketide complex to these second proteins occurs through the dissimilar regions of the drugs known as the "effector" domains.



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The three component FKBP-polyketide-effector complex is required for signal transduction and subsequent immunosuppressive activity of FK-506, FK-520, and rapamycin. Modifications in the effector domains of FK-506, FK-520, and rapamycin that destroy binding to the effector proteins (calcineurin or RAFT) lead to loss of immunosuppressive activity, even though FKBP binding is unaffected. Further, such analogs antagonize the immunosuppressive effects of the parent polyketides, because they compete for FKBP. Such non-immunosuppressive analogs also show reduced toxicity (see Dumont et al., 1992, Journal of Experimental Medicine 176, 751-760), indicating that much of the toxicity of these drugs is not linked to FKBP binding.

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In addition to immunosuppressive activity, FK-520, FK-506, and rapamycin have neurotrophic activity. In the central nervous system and in peripheral nerves, immunophilins are referred to as "neuroimmunophilins". The neuroimmunophilin FKBP is markedly enriched in the central nervous system and in peripheral nerves. Molecules that bind to the neuroimmunophilin FKBP, such as FK-506 and FK-520, have the remarkable effect of stimulating nerve growth. *In vitro*, they act as neurotrophins, i.e., they promote neurite outgrowth in NGF-treated PC12 cells and in sensory neuronal cultures, and in intact animals, they promote regrowth of damaged facial and sciatic nerves, and repair lesioned serotonin and dopamine neurons in the brain. See Gold *et al.*, Jun. 1999, *J. Pharm. Exp. Ther.* 289(3): 1202-1210; Lyons *et al.*, 1994, *Proc. National Academy of Science* 91: 3191-3195; Gold *et al.*, 1995, *Journal of Neuroscience* 15:

7509-7516; and Steiner et al., 1997, Proc. National Academy of Science 94: 2019-2024. Further, the restored central and peripheral neurons appear to be functional.

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Compared to protein neurotrophic molecules (BNDF, NGF, etc.), the small-molecule neurotrophins such as FK-506, FK-520, and rapamycin have different, and often advantageous, properties. First, whereas protein neurotrophins are difficult to deliver to their intended site of action and may require intra-cranial injection, the small-molecule neurotrophins display excellent bioavailability; they are active when administered subcutaneously and orally. Second, whereas protein neurotrophins show quite specific effects, the small-molecule neurotrophins show rather broad effects. Finally, whereas protein neurotrophins often show affects on normal sensory nerves, the small-molecule neurotrophins do not induce aberrant sprouting of normal neuronal processes and seem to affect damaged nerves specifically. Neuroimmunophilin ligands have potential therapeutic utility in a variety of disorders involving nerve degeneration (e.g. multiple sclerosis, Parkinson's disease, Alzheimer's disease, stroke, traumatic spinal cord and brain injury, peripheral neuropathies).

Recent studies have shown that the immunosuppressive and neurite outgrowth activity of FK-506, FK-520, and rapamycin can be separated; the neuroregenerative activity in the absence of immunosuppressive activity is retained by agents which bind to FKBP but not to the effector proteins calcineurin or RAFT. See Steiner *et al.*, 1997, *Nature Medicine 3*: 421-428.



Available structure-activity data show that the important features for neurotrophic activity of rapamycin, FK-520, and FK-506 lie within the common, contiguous segments of the macrolide ring that bind to FKBP. This portion of the molecule is termed the "FKBP binding domain" (see VanDuyne *et al.*, 1993, *Journal of Molecular Biology 229*: 105-124.). Nevertheless, the effector domains of the parent macrolides contribute to conformational rigidity of the binding domain and thus indirectly contribute to FKBP binding.

"FKBP binding domain"

There are a number of other reported analogs of FK-506, FK-520, and rapamycin that bind to FKBP but not the effector protein calcineum or RAFT. These analogs show effects on nerve regeneration without immunosuppressive effects.

Naturally occurring FK-520 and FK-506 analogs include the antascomycins, which are FK-506-like macrolides that lack the functional groups of FK-506 that bind to calcineurin (see Fehr *et al.*, 1996, *The Journal of Antibiotics* 49: 230-233). These molecules bind FKBP as effectively as does FK-506; they antagonize the effects of both FK-506 and rapamycin, yet lack immunosuppressive activity.

Antascomycin A

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Other analogs can be produced by chemically modifying FK-506, FK-520, or rapamycin. One approach to obtaining neuroimmunophilin ligands is to destroy the effector binding region of FK-506, FK-520, or rapamycin by chemical modification. While the chemical modifications permitted on the parent compounds are quite limited, some useful chemically modified analogs exist. The FK-520 analog L-685,818 (ED₅₀ = 0.7 nM for FKBP binding; see Dumont *et al.*, 1992), and the rapamycin analog WAY-124,466 (IC₅₀ = 12.5 nM; see Ocain *et al.*, 1993, *Biochemistry Biophysical Research Communications 192*: 1340-134693) are about as effective as FK-506, FK-520, and rapamycin at promoting neurite outgrowth in sensory neurons (see Steiner *et al.*, 1997).

L-005,818 VVAY-124,400

One of the few positions of rapamycin that is readily amenable to chemical modification is the allylic 16-methoxy group; this reactive group is readily exchanged by acid-catalyzed nucleophilic substitution. Replacement of the 16-methoxy group of rapamycin with a variety of bulky groups has produced analogs showing selective loss of immunosuppressive activity while retaining FKBP-binding (see Luengo *et al.*, 1995, *Chemistry & Biology 2*: 471-481). One of the best compounds, 1, below, shows complete loss of activity in the splenocyte proliferation assay with only a 10-fold reduction in binding to FKBP.

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There are also synthetic analogs of FKBP binding domains. These compounds reflect an approach to obtaining neuroimmunophilin ligands based on "rationally designed" molecules that retain the FKBP-binding region in an appropriate conformation for binding to FKBP, but do not possess the effector binding regions. In one example, the ends of the FKBP binding domain were tethered by hydrocarbon chains (see Holt *et al.*, 1993, *Journal of the American Chemical Society 115*: 9925-9938); the best analog, 2, below, binds to FKBP about as well as FK-506. In a similar approach, the ends of the FKBP binding domain were tethered by a tripeptide to give analog 3, below, which binds

to FKBP about 20-fold poorer than FK-506. These compounds are anticipated to have neuroimmunophilin binding activity.

In a primate MPTP model of Parkinson's disease, administration of FKBP ligand GPI-1046 caused brain cells to regenerate and behavioral measures to improve. MPTP is a neurotoxin, which, when administered to animals, selectively damages nigral-striatal dopamine neurons in the brain, mimicking the damage caused by Parkinson's disease. Whereas, before treatment, animals were unable to use affected limbs, the FKBP ligand restored the ability of animals to feed themselves and gave improvements in measures of locomotor activity, neurological outcome, and fine motor control. There were also corresponding increases in regrowth of damaged nerve terminals. These results demonstrate the utility of FKBP ligands for treatment of diseases of the CNS.

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From the above description, two general approaches towards the design of non-immunosuppressant, neuroimmunophilin ligands can be seen. The first involves the construction of constrained cyclic analogs of FK-506 in which the FKBP binding domain is fixed in a conformation optimal for binding to FKBP. The advantages of this approach are that the conformation of the analogs can be accurately modeled and predicted by computational methods, and the analogs closely resemble parent molecules that have proven pharmacological properties. A disadvantage is that the difficult chemistry limits the numbers and types of compounds that can be prepared. The second approach involves the trial and error construction of acyclic analogs of the FKBP binding domain by conventional medicinal chemistry. The advantages to this approach are that the chemistry is suitable for production of the numerous compounds needed for such interactive chemistry-bioassay approaches. The disadvantages are that the molecular types of compounds that have emerged have no known history of appropriate pharmacological properties, have rather labile ester functional groups, and are too conformationally mobile to allow accurate prediction of conformational properties.

The present invention provides useful methods and reagents related to the first approach, but with significant advantages. The invention provides recombinant PKS

genes that produce a wide variety of polyketides that cannot otherwise be readily synthesized by chemical methodology alone. Moreover, the present invention provides polyketides that have either or both of the desired immunosuppressive and neurotrophic activities, some of which are produced only by fermentation and others of which are produced by fermentation and chemical modification. Thus, in one aspect, the invention provides compounds that optimally bind to FKBP but do not bind to the effector proteins. The methods and reagents of the invention can be used to prepare numerous constrained cyclic analogs of FK-520 in which the FKBP binding domain is fixed in a conformation optimal for binding to FKBP. Such compounds will show neuroimmunophilin binding (neurotrophic) but not immunosuppressive effects. The invention also allows direct manipulation of FK-520 and related chemical structures via genetic engineering of the enzymes involved in the biosynthesis of FK-520 (as well as related compounds, such as FK-506 and rapamycin); similar chemical modifications are simply not possible because of the complexity of the structures. The invention can also be used to introduce "chemical handles" into normally inert positions that permit subsequent chemical modifications.

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Several general approaches to achieve the development of novel neuroimmunophilin ligands are facilitated by the methods and reagents of the present invention. One approach is to make "point mutations" of the functional groups of the parent FK-520 structure that bind to the effector molecules to eliminate their binding potential. These types of structural modifications are difficult to perform by chemical modification, but can be readily accomplished with the methods and reagents of the invention.

A second, more extensive approach facilitated by the present invention is to utilize molecular modeling to predict optimal structures *ab initio* that bind to FKBP but not effector molecules. Using the available X-ray crystal structure of FK-520 (or FK-506) bound to FKBP, molecular modeling can be used to predict polyketides that should optimally bind to FKBP but not calcineurin. Various macrolide structures can be generated by linking the ends of the FKBP-binding domain with "all possible" polyketide chains of variable length and substitution patterns that can be prepared by genetic manipulation of the FK-520 or FK-506 PKS gene cluster in accordance with the methods of the invention. The ground state conformations of the virtual library can be determined, and compounds that possess binding domains most likely to bind well to FKBP can be prepared and tested.

Once a compound is identified in accordance with the above approaches, the invention can be used to generate a focused library of analogs around the lead candidate, to "fine tune" the compound for optimal properties. Finally, the genetic engineering methods of the invention can be directed towards producing "chemical handles" that enable medicinal chemists to modify positions of the molecule previously inert to chemical modification. This opens the path to previously prohibited chemical optimization of lead compounds by time-proven approaches.

Moreover, the present invention provides polyketide compounds and the recombinant genes for the PKS enzymes that produce the compounds that have significant advantages over FK-506 and FK-520 and their analogs. The metabolism and pharmacokinetics of tacrolimus has been exampled studied, and FK-520 is believed to be similar in these respects. Absorption of tacrolimus is rapid, variable, and incomplete from the gastrointestinal tract (Harrison's Principles of Internal Medicine, 14th edition, 1998, McGraw Hill, 14, 20, 21, 64-67). The mean bioavailability of the oral dosage form is 27%, (range 5 to 65%). The volume of distribution (VolD) based on plasma is 5 to 65 L per kg of body weight (L/kg), and is much higher than the VolD based on whole blood concentrations, the difference reflecting the binding of tacrolimus to red blood cells. Whole blood concentrations may be 12 to 67 times the plasma concentrations. Protein binding is high (75 to 99%), primarily to albumin and alpha1-acid glycoprotein. The half-life for distribution is 0.9 hour; elimination is biphasic and variable: terminal-11.3 hr (range, 3.5 to 40.5 hours). The time to peak concentration is 0.5 to 4 hours after oral administration.

Tacrolimus is metabolized primarily by cytochrome P450 3A enzymes in the liver and small intestine. The drug is extensively metabolized with less than 1% excreted unchanged in urine. Because hepatic dysfunction decreases clearance of tacrolimus, doses have to be reduced substantially in primary graft non-function, especially in children. In addition, drugs that induce the cytochrome P450 3A enzymes reduce tacrolimus levels, while drugs that inhibit these P450s increase tacrolimus levels.

Tacrolimus bioavailability doubles with co-administration of ketoconazole, a drug that inhibits P450 3A. See, Vincent *et al.*, 1992, *In vitro* metabolism of FK-506 in rat, rabbit, and human liver microsomes: Identification of a major metabolite and of cytochrome P450 3A as the major enzymes responsible for its metabolism, *Arch. Biochem. Biophys.* 294: 454-460; Iwasaki *et al.*, 1993, Isolation, identification, and biological activities of oxidative metabolites of FK-506, a potent immunosuppressive macrolide lactone, *Drug Metabolism & Disposition 21*: 971-977; Shiraga *et al.*, 1994, Metabolism of FK-506, a

potent immunosuppressive agent, by cytochrome P450 3A enzymes in rat, dog, and human liver microsomes, *Biochem. Pharmacol.* 47: 727-735; and Iwasaki et al., 1995, Further metabolism of FK-506 (Tacrolimus); Identification and biological activities of the metabolites oxidized at multiple sites of FK-506. *Drug Metabolism & Disposition* 23: 28-34. The cytochrome P450 3A subfamily of isozymes has been implicated as important in this degradative process.

Structures of the eight isolated metabolites formed by liver microsomes are shown in Figure 6. Four metabolites of FK-506 involve demethylation of the oxygens on carbons 13, 15, and 31, and hydroxylation of carbon 12. The 13-demethylated (hydroxy) compounds undergo cycnizations of the 13-hydroxy at C-10 to give MI, MVI and MVII, and the 12-hydroxy metabolite at C-10 to give I. Another four metabolites formed by oxidation of the four metabolites mentioned above were isolated by liver microsomes from dexamethasone treated rats. Three of these are metabolites doubly demethylated at the methoxy groups on carbons 15 and 31 (M-V), 13 and 31 (M-VI), and 13 and 15 (M-VII). The fourth, M-VIII, was the metabolite produced after demethylation of the 31-methoxy group, followed by formation of a fused ring system by further oxidation. Among the eight metabolites, M-II has immunosuppressive activity comparable to that of FK-506, whereas the other metabolites exhibit weak or negligible activities. Importantly, the major metabolite of human, dog, and rat liver microsomes is the 13-demethylated and cyclized FK-506 (M-I).

Thus, the major metabolism of FK-506 proceeds via 13-demethylation followed by cyclization to the inactive M-I, this representing about 90% of the metabolic products after a 10 minute incubation with liver microsomes. Analogs of tacrolimus that do not possess a C-13 methoxy group would not be susceptible to the first and most important biotransformation in the destructive metabolism of tacrolimus (i.e. cyclization of 13-hydroxy to C-10). Thus, a 13-desmethoxy analog of FK-506 should have a longer half-life in the body than does FK-506. The C-13 methoxy group is believed not to be required for binding to FKBP or calcineurin. The C-13 methoxy is not present on the identical position of rapamycin, which binds to FKBP with equipotent affinity as tacrolimus. Also, analysis of the 3-dimensional structure of the FKBP-tacrolimus-calcineurin complex shows that the C-13 methoxy has no interaction with FKBP and only a minor interaction with calcineurin. The present invention provides C-13-desmethoxy analogs of FK-506 and FK-520, as well as the recombinant genes that encode the PKS enzymes that catalyze their synthesis and host cells that produce the compounds.

These compounds exhibit, relative to their naturally occurring counterparts, prolonged immunosuppressive action *in vivo*, thereby allowing a lower dosage and/or reduced frequency of administration. Dosing is more predictable, because the variability in FK-506 dosage is largely due to variation of metabolism rate. FK-506 levels in blood can vary widely depending on interactions with drugs that induce or innibit cytochrome P450 3A (summarized in USP Drug Information for the Health Care Professional). Of particular importance are the numerous drugs that inhibit or compete for CYP 3A, because they increase FK-506 blood levels and lead to toxicity (Prograf package insert, Fujisawa TUS, Rev 4/97, Rec 6/97). Also important are the drugs that induce P450 3A (e.g. Dexage Lone), because they decrease FK-506 blood levels and reduce efficacy. Because the major site of CYP 3A action on FK-506 is removed in the analogs provided by the present invention, those analogs are not as susceptible to drug interactions as the naturally occurring compounds.

Hyperglycemia, nephrotoxicity, and neurotoxicity are the most significant
adverse effects resulting from the use of FK-506 and are believed to be similar for FK520. Because these effects appear to occur primarily by the same mechanism as the
immunosuppressive action (i.e. FKBP-calcineurin interaction), the intrinsic toxicity of
the desmethoxy analogs may be similar to FK-506. However, toxicity of FK-506 is dose
related and correlates with high blood levels of the drug (Prograf package insert,
Fujisawa US, Rev 4/97, Rec 6/97). Because the levels of the compounds provided by
the present invention should be more controllable, the incidence of toxicity should be
significantly decreased with the 13-desmethoxy analogs. Some reports show that certain
FK-506 metabolites are more toxic than FK-506 itself, and this provides an additional
reason to expect that a CYP 3A resistant analog can have lower toxicity and a higher
therapeutic index.

Thus, the present invention provides novel compounds related in structure to FK-506 and FK-520 but with improved properties. The invention also provides methods for making these compounds by fermentation of recombinant host cells, as well as the recombinant host cells, the recombinant vectors in those host cells, and the recombinant proteins encoded by those vectors. The present invention also provides other valuable materials useful in the construction of these recombinant vectors that have many other important applications as well. In particular, the present invention provides the FK-520 PKS genes, as well as certain genes involved in the biosynthesis of FK-520 in recombinant form.

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FK-520 is produced at relatively low levels in the naturally occurring cells, *Streptomyces hygroscopicus* var. *ascomyceticus*, in which it was first identified. Thus, another benefit provided by the recombinant FK-520 PKS and related genes of the present invention is the ability to produce FK-520 in greater quantities in the recombinant host cells provided by the invention. The invention also provides methods for making novel FK-520 analogs, in addition to the desmethoxy analogs described above, and derivatives in recombinant host cells of any origin.

The biosynthesis of FK-520 involves the action of several enzymes. The FK-520 PKS enzyme, which is composed of the *fkbA*, *fkbB*, *fkbC*, and *fkbP* gene products, synthesizes the core structure of the molecule. There is also a hydroxylation at C-9 mediated by the P450 hydroxylase that is the *fkbD* gene product and that is oxidized by the *fkbO* gene product to result in the formation of a keto group at C-9. There is also a methylation at C-31 that is mediated by an O-methyltransferase that is the *fkbM* gene product. There are also methylations at the C-13 and C-15 positions by a methyltransferase believed to be encoded by the fkbG gene; this methyltransferase may act on the hydroxymalonyl CoA substrates prior to binding of the substrate to the AT domains of the PKS during polyketide synthesis. The present invention provides the genes encoding these enzymes in recombinant form. The invention also provides the genes encoding the enzymes involved in ethylmalonyl CoA and 2-hydroxymalonyl CoA biosynthesis in recombinant form. Moreover, the invention provides *Streptomyces hygroscopicus* var. *ascomyceticus* recombinant host cells lacking one or more of these genes that are useful in the production of useful compounds.

The cells are useful in production in a variety of ways. First, certain cells make a useful FK-520-related compound merely as a result of inactivation of one or more of the FK-520 biosynthesis genes. Thus, by inactivating the C-31 O-methyltransferase gene in *Streptomyces hygroscopicus* var. *ascomyceticus*, one creates a host cell that makes a desmethyl (at C-31) derivative of FK-520. Second, other cells of the invention are unable to make FK-520 or FK-520 related compounds due to an inactivation of one or more of the PKS genes. These cells are useful in the production of other polyketides produced by PKS enzymes that are encoded on recombinant expression vectors and introduced into the host cell.

Moreover, if only one PKS gene is inactivated, the ability to produce FK-520 or an FK-520 derivative compound is restored by introduction of a recombinant expression vector that contains the functional gene in a modified or unmodified form. The introduced gene produces a gene product that, together with the other endogenous and

functional gene products, produces the desired compound. This methodology enables one to produce FK-520 derivative compounds without requiring that all of the genes for the PKS enzyme be present on one or more expression vectors. Additional applications and benefits of such cells and methodology will be readily apparent to those of skill in the art after consideration of how the recombinant genes were isolated and employed in the construction of the compounds of the invention.

The FK-520 biosynthetic genes were isolated by the following procedure. Genomic DNA was isolated from *Streptomyces hygroscopicus* var. *ascomyceticus* (ATCC 14891) using the lysozyme/proteinase K protocol described in Genetic Manipulation of *Streptomyces* - A Laboratory Manual (Hopwood *et al.*, 1986). The average size of the DNA was estimated to be between 80 - 120 kb by electrophoresis on 0.3% agarose gels. A library was constructed in the SuperCosTM vector according to the manufacturer's instructions and with the reagents provided in the commercially available kit (Stratagene). Briefly, 100 µg of genomic DNA was partially digested with 4 units of *Sau*3A I for 20 min. in a reaction volume of 1 mL, and the fragments were dephosphorylated and ligated to SuperCos vector arms. The ligated DNA was packaged and used to infect log-stage XL1-BlueMR cells. A library of about 10,000 independent cosmid clones was obtained.

Based on recently published sequence from the FK-506 cluster (Motamedi and Shafiee, 1998, *Eur. J. Biochem. 256*: 528), a probe for the *fkbO* gene was isolated from ATCC 14891 using PCR with degenerate primers. With this probe, a cosmid designated pKOS034-124 was isolated from the library. With probes made from the ends of cosmid pKOS034-124, an additional cosmid designated pKOS034-120 was isolated. These cosmids (pKOS034-124 and pKOS034-120) were shown to contain DNA inserts that overlap with one another. Initial sequence data from these two cosmids generated sequences similar to sequences from the FK-506 and rapamycin clusters, indicating that the inserts were from the FK-520 PKS gene cluster. Two *Eco*RI fragments were subcloned from cosmids pKOS034-124 and pKOS034-120. These subclones were used to prepare shotgun libraries by partial digestion with *Sau*3AI, gel purification of fragments between 1.5 kb and 3 kb in size, and ligation into the pLitmus28 vector (New England Biolabs). These libraries were sequenced using dye terminators on a Beckmann CEQ2000 capillary electrophoresis sequencer, according to the manufacturer's protocols.

To obtain cosmids containing sequence on the left and right sides of the sequenced region described above, a new cosmid library of ATCC 14891 DNA was prepared essentially as described above. This new library was screened with a new fkbM

probe isolated using DNA from ATCC 14891. A probe representing the *fkbP* gene at the end of cosmid pKOS034-124 was also used. Several additional cosmids to the right of the previously sequenced region were identified. Cosmids pKOS065-C31 and pKOS065-C3 were identified and then mapped with restriction enzymes. Initial sequences from these cosmids were consistent with the expected organization of the cluster in this region. More extensive sequencing showed that both cosmids contained in addition to the desired sequences, other sequences not contiguous to the desired sequences on the host cell chromosomal DNA. Probing of additional cosmid libraries identified two additional cosmids, pKOS065-M27 and pKOS065-M21, that contained the desired sequences in a contiguous segment of chromosomal DNA. Cosmids pKOS034-124, pKOS034-120, pKOS065-M27, and pKOS065-M21 have been deposited with the American Type Culture Collection, Manassas, VA, USA. The complete nucleotide sequence of the coding sequences of the genes that encode the proteins of the FK-520 PKS are shown below but can also be determined from the cosmids of the invention deposited with the ATCC using standard methodology.

Referring to Figures 1 and 3, the FK-520 PKS gene cluster is composed of four open reading frames designated fkbB, fkbC, fkbA, and fkbP. The fkbB open reading frame encodes the loading module and the first four extender modules of the PKS. The fkbC open reading frame encodes extender modules five and six of the PKS. The fkbA open reading frame encodes extender modules seven, eight, nine, and ten of the PKS. The fkbP open reading frame encodes the NRPS of the PKS. Each of these genes can be isolated from the cosmids of the invention described above. The DNA sequences of these genes are provided below preceded by the following table identifying the start and stop codons of the open reading frames of each gene and the modules and domains contained therein.

	Nucleotides complement (412 - 1836)	Gene or Domain fkbW
	complement (2020 - 3579)	fkbV
30	complement (3969 - 4496)	fkbR2
	complement (4595 - 5488)	fkbR1
	5601 - 6818	fkbE
	6808 - 8052	fkbF
	8156 - 8824	fkbG
35	complement (9122 - 9883)	fkbH
	complement (9894 - 10994)	fkbI
	complement (10987 - 11247)	fkbJ
	complement (11244 - 12092)	fkbK
	complement (12113 - 13150)	fkbL
40	complement (13212 - 23988)	fkbC

	complement (23992 - 46573)	fkbB
	46754 - 47788	fkbO
	47785 - 52272	fkbP
	52275 - 71465	fkbA
5	71462 - 72628	fkbD
	72625 - 73407	fkbM
	complement (73460 - 76202)	fkbN
	complement (76336 - 77080)	fkbQ
	complement (77076 - 77535)	fkbS
10	complement (44974 - 46573)	CoA ligase of loading domain
	complement (43777 - 44629)	ER of loading domain
	complement (43144 - 43660)	ACP of loading domain
	complement (41842 - 43093)	KS of extender module 1 (KS1)
	complement(40609 - 41842)	AT1
15	complement (39442 - 40609)	DH1
	complement (38677 - 39307)	KR1
	complement (38371 - 38581)	ACP1
	complement (37145 - 38296)	KS2
20	complement (35749 - 37144)	AT2
20	complement (34606 - 35749)	DH2 (inactive)
	complement (33823 - 34480)	KR2
	complement (33505 - 33715)	ACP2
	complement (32185 - 33439)	KS3
25	complement (31018 - 32185)	AT3
<i>43</i>	complement (29869 - 31018) complement (29092 - 29740)	DH3 (inactive) KR3
	complement (28750 - 28960)	ACP3
	complement (27430 - 28684)	KS4
	complement (26146 - 27430)	AT4
30	complement (24997 - 26146)	DH4 (inactive)
	complement (24163 - 24373)	ACP4
	complement (22653 - 23892)	KS5
	complement (21420 - 22653)	AT5
	complement (20241 - 21420)	DH5
35	complement (19464 - 20097)	KR5
	complement (19116 - 19326)	ACP5
	complement (17820 - 19053)	KS6
	complement (16587 - 17820)	AT6
	complement (15438 - 16587)	DH6
40	complement (14517 - 15294)	ER6
	complement (13761 - 14394)	KR6
	complement (13452 - 13662)	ACP6
	5236? - 53576	KS7
4.5	53577 - 54716	AT7
45	54717 - 55871	DH7
	56019 - 56819	ER7
	56943 - 57575	KR7
	57710 - 57920 57900 - 50242	ACP7
50	57990 - 59243 50244 - 60208	KS8
50	59244 - 60398 60399 - 61412	AT8
	61548 - 62180	DH8 (inactive) KR8
	01240 - 02100	VIVO

	62328	- 62537		ACP8			
		- 63854		KS9			
				AT9			
		- 65084					
		- 66254		DH9			
5		- 67175		ER9			
		- 67931		KR9			
		- 68303		ACP9			
	68397	- 69653		KS10			
	69654	- 70985		AT10			
10	71064	- 71273		ACP10			
	_			0010000100	0000000000	000000000000000000000000000000000000000	000000000000000000000000000000000000000
		GATOTCAGGQ TGTACGGACC		GOGGOGATTG		GTGAAGACCT	CGCCGCTGCT
	• 44	TTACAAGATC	CTCACATTGC	GCGACCGCCA		- STMSCOMCA	GAGGCAAACC
15	181	GAAAGGGCGC	GGGGGGTCCG	CACCATAGE	. A0010A	CGAGAGTGGC	GCACCCGCGC
	241	ACCGTCACCT	CTCTCCCCCG	CCGGCGGGAT	3000330303	ACGGTTGG	GCTCTCC .CG
	301	ACGCTGAACA	CCCGCGCGGT	GTGGCGTCGG	GGACACCGTG	TGGCATCGGC	CGGGTGACGG
	361	TACGGGGAGG	GCGTACGGCG	GCCGTGGCTC	GTGGTGAGGG	casacagaaa	GTCATCCGTC
2.0	421	GAGACGGCAC	TOGGCGAGCA	GGGACGCCTG	GTCGGCACIT	GOGGGCCGGA	CGACCGTGTG
20	481	GTTCGCGGGC	GGGCGGTGGC	CGSTGGTGAG	COAGGTCTCC		AGGCTGAGCG
	4.	GTGACACGGC	AGCAMAGGCC	GBAGTCGGTC	33334A3313	TOGROGAGGG	CGTCGGTGTG
	6	COTTGCCGTCC	TOGATGCGGT	naimalaaim		390000TGCC	GGACATACGC
	721	GUGTACACGT CAGCGGCTTG	CCGATACGAC	COGGCANCOC		ADGGCGCGT	GGACGCUGGA
25	781			AGTCGGCATC	GCAGCCGGG	ACCGTCCCCG	GGGCGCAATA
	841	CGGTGTGCCG	GCTTCCTTCT	CCCCATCGAA	gddggggtdg	AACTCCTCGC	GGTAGACGCG
	901	CTGCGTCAGA	TCCCAGTAGA	CCTCGTGGTG	GTACGGCCAC	AAGAACTOGG	AGTCGGCCGG
	961	GAACCCGGCG	CGGAGCAGCG		CTGGCCGGCT	GOGGGGGCGGC	CTGCCGCGTA
20	1021	GGTGGGGTAG				AGGTTGGGAC	
30	1081		CCTTCCCAGT			TCGGGATGGT	TCTCCAGCTG
	114' 1201		AGGTAGCCGC			AGGGTGCGCT AGCTGGGTGA	
	1261	GTGGTAGCGC CCACTCGGCG	TGGGCGACCG ACGGCGTCGC	ACGCGCGGGC		TAGAACGCGG	GGCCGGTGTT
	1321	GCCCTTGTCG		AGGCGTAACC	GCGGGGCGAGC	ACCCAGTOGG	CGATGGCCCG
35	1381	STCSTTSGCG		GGTTACCGGG	ggrgggggg	ACGACCAGGC	CACCGTTCCA
	1441	GCGGTCGGGC	AGCCGGATGA	CGAACTGGGC	GTCGTGGTTC	CACCCGTGGT	TGGTGTTGGT
	1501		TCGGGGAAGT			GGCACTCCGG	
	1561					GTGTGGCCGG	
• ()	1621					CGTGCCGCCG	
40	1681	CGGTGAGGGG	AGACGGGGGG	AG FGACCGTC		GGAGCAGGCC	CCCTCCCTCC
		TCTCGGGGCC					
		GATGACGGAC					
	1921	ACTGAGGCCC	CTCAGAGGTG	GGCCGCCGCC	ATGACGGGGG	CGGGACCGCG	GGCGCTCCGG
45	1981	GGCGG IGCCC	GCGGCCGCCA	CCGGTTCCGG	GTCCCCGGGT	CAGGGACAGG	TGTCGTTCGC
	2041	GACGGTGAAG	TAGCCGGTCG	GCGACTCTTT	CAAGGTGGTC	GTGACGAAGG	TGTTGTACAG
		GCCCATGTTC					
		CGCCTGGACG					
50		CGCGGTGACC					
30	2281	GIAGGIGIGC				TACGACGTCG	
	2001	GGTCAGGCTG	ATROTROTOT	CONTROCCC	GGTGGGGGG	+GGCCGGACG	GAGCGGGCAG
	2461	CGAACCGGGG	TCGGAGGCGG	ATCCGCTCAG	GCCGAAGAAC	TGCGTGATCC	AGTAGCTGGA
	2521	ACAGATCGAG	TCCAGGAAGT	AGGCGGCGCC	ggTgcTgccs	CACTGCTGTG	CTCCGGTGCC
55	2581					TTCACCTCCA	
		TCCGTCCGCG					
	2701	GTCCGGCGTC	TGGGACACGC	CGTGCACAGC	GGTOCACTGG	TCGCGCAACT	CGTCGGCGTT
	2761	GCGCGCGCG	ACGGTGGTGT	CCTTGTCGCC	GTGCCAGATG	GCCACGCGCG	GCCACGGGCC
60		CGACCACGAG					
60	1081	CCCGGGGTTC GGCGACGACC	ATGUACAGGT	ACGUGUTGUT	endelidene Ogganacene	GCGLGCATCA	CCGACGTCAT
	794I	JURUJAUGAUG	000000000	SUMMUNCULU	COOMIMOSIS	GOGROURICA	COORDGICAL

		GGCACCGCCG				GGGTCCGCGC	CGTAGGCGGA
	30€1	GACGGTGTGA	GCGGCCATCT	GCCGGATCGA	CGCGGGCTTCG	CCCTGGCCCC	TGCGGTTGTC
	3121	Gergererag	AACCAGTTGA	ASCACCTSTT	CGCGTTGTTC	GACGACGIGG	TCTCGGCGAA
	3191	CACGAGCAGG		GGTCCGCGAA		COGGAGTTGT	CGGCGTAGCC
5	2031		TGGGTGGAAC	CSTSCAGSGD	3110100100	GCCGGCTCCG	
-	3331			TGTTCAGCCG	222222222	GTGCCGAAGT	
			TAGACGTACA		2000333.10		
	3361			GAJCGGGGTT	3333M33356	SCCGCGGCGT	
	3421	caccinacca				ACGGCCACGA	
	3481	CACCCCCCGC	CGTCCCGGAC			GGCGAGGAGG	
10	3541	CAGCGGGGTG	AGGATTCCCC	GGAACGGCGG	CGGCTGCATG	GOGGCTCCCT	CGATGTCGTG
	3601	GGGGGGACAC	GGAGGGCTCC	CTGACGTCGA	TCAGTGGGAG	CGCCCCGGTG	CCCGGCACCG
	3661	TAGGGGTGGT	TCAACCCGCA	ACGGTATGGC	CCGGAGCACC	ACACCCCGCA	CCGCGCGATG
	3701	TGCGCCCGGA		CGCCTTGCGG		CCGGACGCGA	
				CATGGTGTCC		GTCGGCCTTG	
1.5			GGTAGGGGGT				
15		ACGGACCGGG				CGGTATGGCG	
	3901	CCAGCCGCGT		CGCCCAAGTG		ACCGTGGCCG	
	3961	CGGRCCGGTC			CGGGACCGCT	DETCCCAGAC	GGGTTCCACC
	-321	GOGGCGAACI	0000100010	TOLDOGGGGG	TAGACCATCA	STGTCCGCTC	GAAGGTGATG
	4081	ACGATGACAC	CGTCCTGGTT	GTAGCCGATG	GTGCGCACGC	TGATGATGCC	TACGTCAGGT
20	4141	CGGCTGGCGG		GTTCAGGACC	TOGGRACIGOS	AGTAGATGGT	GTCGCCCTCG
				GACCCGGTCC	CAGCCGAGGT	TGGCCATCAC	
		ATGTCGGTGA				AGGTGGAGTC	
	4261					GCAGCGGCGC	
					CGGTCGAAGT		
	4381	GTCAGGAGCG	TGAGCCAGGA	GTTGTCGGTC	Luumaamaaa	TSCGGCCCAG	
25	4441			GTCCTCGAAG	TAGOGGCCCT	GCCAGCCCTC	
	4501	GTGCGGGTGG	CGTCCTGGTC	CGGGTTCTCA	GTCGTCATGG	COCTCATTCT	GGGAAGTCCC
	4561	CGGTCCGCTG	TGAAATGCCG	AACCTTCACC	GGGCTCATAC	GTGCGGCGCA	TGAGCCCTGG
	4621	ACCGTACGTA	GTCGTAGAAC	CTCGCCACCA	CTGGCGCGCG	TGGTCCTCCG	GCGAGTGTGA
		CCACGCCGAC		GCCTGCGGGT		CACGGCGACG	GCGTGGTCAC
30	4741					ACCGAGGCCG	GCGGCGACCA
50	4801						
		CGGCGCACAG					
		CCTCATCGGC					
	4921	CCTCATCGGC	CAGCTCCGCG	GTCCGCACCC	GGCGGCG.CI	GGCCAGCCGG	TGICCGGGIG
3.5	4981	GGACGAGCAG	GCACAGTGCC	TCGTCCCGCA	GIGGIGICUA	CTCCACATCG	1 CCCCCGGCGG
35	5041	GTCGTGGGCT	GGTCAGCCCC	AGGTCCAGCC	TGCTGTTGUG	GACGTCGTCG	ACCACGGCGT
	5101		GCCGCGCAGT	TCGAAGGTGG	TGCCGGGAGC	CAGCCGGCGG	TACCCGGCGA
	5161	GGAGGTCGGG	CACCAGCCAG	GTGCCGTAGG	AGTGCAGGAA	ACCCAGTGCC	ACGGTGCCGG
	5221	TGTCGGGGTC	GATCAGGGCG	GTGATGCGCT	GCTCGGCGCC	GGAGACCTCA	CTGATCGCGC
	5281	GCAGGGCGTG	GGCGCGGAAG	ACCTCGCCGT	ACTTGTTGAG	CCGGAGCCGG	TTCTGGTGCC
40	6341	GGTCGAACAG	CGGCACGCCC	ACTOGTOGOT	nnaggogoog	SATGGCCCTG	GACAGGGTCG
10	5101	GCTGGGAGAT	CEMCA COCCE	TOCCOCCOCI	TRATCLOSTS	OTCOTOCTO	GCCAAGGCCG
	5161	TGAACCACTG	GIIGAGCCGI	100000010A	10010110010	CCTACCCCC	ATCCTCCTCC
	2401	IGAACCACIG	CAACICCCGI	ATCTCCATGC	AGGGACIAIA	COLACCOCC	Chechaeche
		CGAGGTTTCG					
	5581	GACCCCATGG	GAGGGACCCC	ATGTCCGAGC	GethTutius	JCCTGAACAG	GAACGCCCCG
45	5641	CCGGGCCCCT	GTCCGGTCTG	CTCGTGGTTT	CTTTGGAGCA	GGCCGTCGCC	GCTCCGTTCG
	5701	CCACCCGCCA	CCTGGCGGAC	CTGGGCGCCC	GTGTCATCAA	GATCGAACGC	CCCGGCAGCG
	5761	GCGACCTCGC	CCGCGGCTAC	GACCGCACGG	TGCGTGGCAT	GTCCAGCCAC	TTCGTCTGGC
	5821	TGAACCGGGG	GAAGGAGAGC	GTCCAGCTCG	ATGTGCGCTC	GCCGGAGGGC	AACCGGCACC
	5881	TGCACGCCTT	GGTGGACCGG	GCCGATGTCC	TGGTGCAGAA	TCTGGCACCC	GGCGCCGCGG
50	5941	GCCGCCTGGC	ATCGGCCACC	AGGTCCTCGC	GCGGAGCCAC	CGAGGCTGAT	CACCTGCGGA
50	2001	CATATCCGGC	TI CCCCCICE	CCCCCTCCTA	COCCEGACCE	CLAGGGGTAC	GACCTCCTGG
	(3.61	TCCAGTGCGA	TACGGCAGTA	CCGGCTGCTA	CCCCCACCCC	CCACACCCC	TOCANGETEE
	1000	ICCAGIGCGA	MGCGGGGCTG	GIGICCALCA	CCGGCACCCC	CTCCCCCTCC	CECAACCCCC
	6121	GCCTGTCCAT	CGCGGACATO	TGTGCGGGA	1G:ACGCG.A	CICCGGCAIC	CTCACGGCCC
	6181	TGCTGAAGCG	GGCCCGCACC	GGCCGGGGCT	CGCAGTIGGA	GGTCTCGATG	CTUGAAGUUC
55	6241	TCGGTGAATG	GATGGGATAC	GCCGAGTACT	ACACGCGCTA	CGGCGGCACC	GCTCCGGCCC
	6301	GCGCCGGCGC	CAGCCACGCG	ACGATCGCCC	CCTACGGCCC	GTTCACCACG	CGCGACGGGC
	6361	AGACGATCAA	TCTCGGGCTC	CAGAACGAGC	GGGAGTGGGC	TICCTTCTGC	GGTGTCGTGC
	6421	TACAACGCCC	CGGTCTCTGC	GACGACCCGC	GCTTTTCCGG	CAACGCCGAC	CGGGTGGCGC
	6181	ACCGCACCGA	GCTCGACGCC	CTGGTGAGCG	AGGTGACGGG	CACGCTCACC	GGCGAGGAAC
60	6211	TGGTGGCGCG	GCTGGAGGAC	GOGTOGATOG	CCTACGCACG	CCAGCGCACC	GTGCGGGAGT
00	2501	TCAGCGAACA	CCCCCX X CTC	CONCRECE	CACGCTGGGC	TCCGTTCGAC	AGCCCGGTCG
	0001	TCAGCGAACA	CCCCCAACIG	CCCCCCCCCCC	COMMONTO	CONCORCO	CCCCCCCCCCCC
	bbbl	GTGCGCTGGA	GGGCCTGATC	CUCUCUGGICA	A CONCOMPOR	CONGUNCTUC	000000000000000000000000000000000000000
	6721	GCCGGGTCCC	GGAGCTGGGC	GAGCATACCG	AGICCGTCCT	GGCGIGGCTG	
	6781	ACAGCGCCGA	CCGCGAAGAG	GCCGGCCATG	CCGAATGAAC	TCACCGGAGT	CCTGATCCTG

				0.000007.00.0		maaaaaaa	
	6841	GCCGCCGTGT	TOCTGCTCGC	CGGCGTACGG	GGGCTGAACA		CGCGCTGGTC
	6901	SCCACCITTO	machagaeu	OFFICERORS	COCARDOCA	COGACGAGGT	SCTGGCGGGT
	6961	TTCCCCGCGA	GCATGTTCCT			TCCTCTTCGG	GATCGCCCGC
£	7021	GTCNACGGCA	COGTOGACTO	33.33.5500.		GGGCGGTGGG	GGCCCGGGTG
5	2.091	OGAGGCGTCC	COTOUNTANT	0		TOTGOGGGAC	AGGCGCGGCC
			1961.590681 Northagae		da digasa da a da a	CGTTCGCCGT	CAGGCACCGC
		AFCGATCOCC	MINIAUSCOSO .	Aufführungen		DOGCAGOOGG	CAGTTTCGCC
	7261	Section of the sectio				AGAAGAACCA	TOTGCCCGTC
10	7321	AGCGGCGGGC	TacTCTTCGC	MODERNOOF	Good and and	TGGCGGTCGC	CSCGGTUTCA
10	7391	TESCHOSTO	TOGGGCGCAG	GOGGOTOSAM	Comunitation	TGGACGAGGA TGATGACGCT	CACCGATCCC
	7441	ANGUAAGGGG	MUUUUUULIIU	000000000	dCGGAAAIAIG	COGGCTTCCT	GACCGCGATG
	7501	GCCGCGCTGG	TGCTGGGAAC	CACGGTCCTC	Control and		GGCCCTCACC
	7561	TIGGCGGCGT	TGCTGGCGCT	GOTOTTCCCC	CGCACCIDUO	AGCAGGCCAC ACGTCGCCCT	CAAGGAGATC GCTCCAGGAG
15	7621	3007GGCCCG	TIGGIGCIGCI	GGTAT GCGGG	ATCGCGGGGGA	TOGGCACCCC	GCTGCTGGCC
13	7661	DINGGERTOG	TGGACTCCCT	00000AA6A13	-memoronana	TOGOCTOGAC	CACCGGGATC
	7747	GCCCTGGTGA	TOTGCTACGT		- Galvanedelina - manamarajam	CCGGTGCCAT	CACCAGGATC
	7801	0.001 MCCMC3	TGATGCCGCT	GTCCGAGGGG		ACGCGAGTCC	CTTCTCCACC
		GGCATGGTGA AATGGTGCTC	TGGCCCTGGC TGGTGGTGGC	G000303303		GGCCCGGCGT	GTACCAGGGG
20	7921 7981			Camadagagas	ONGCODULGO ONGCODUCGO	CGGCCGCCTG	GGCGGCCTTC
±0	8041	TTGCTGTGGT GTGGTGGCGT	GGGGGGGGCGG GAGCGCAGCG	GAGCGGGAAT	CCCCTGGAGC	CCGTTTCCCG	TGCTGTGTCG
	8101	OTGACGTAGC	GTCAAGTCCA	CGTGCCGGGG	GGGCAGTACG	CCTAGCATGT	CGGGCATGGC
	8161	TAATCAGATA		ACACGCTGCT	CGCTTACGTA	CGGAAGGTGT	CCCTGCGCGA
	6221	TGACGAGGTG	CTGAGCCGGC	# CCCCCCCC	GACGGCCGAG	CTGCCGGGCG	GTGGCGTACT
25	5281	GOOGGTGCAG	GCCGAGGAGG	GACAGTTCCC.	CGASTTCCTG	STGCGGTTGA	CCGSCGCGCG
- 3	5341	TOMOGRACIO	GAGAT GGGGA	CSTADACCUS	CTACASCASS	crerecerse	CCCGCGGATT
	8401	GGCCCGGG	GGCCGTGTGG	TSACGTGCGA	TOTOATOOOO	AAGTGGCCCG	AGGTGGGCGA
	8461	GUGGTACTGG	GAGGAGGCCG	GGGTTGCCGA	CCCGATCCAC	GTCCGGATCS	GCGACGUCCG
	8521		ACCGGGCTGC	TOGACGAGGC	GGGCGCGGGG	CCGGAGTCGT	TCGACATGGT
30	8581	GTTCAPCGAC	GCCGACAAGG		CGCCTACTAC	GAGGCGGCGC	TGCCGCTGGT
	8641	ACGCCGCGGC		TOSTOGACAA	CACGCTGTTC	TTCGGCCGGG	TGGCCGACGA
	8701		GACCCGGACA		ACGCGAACTC	AACGCGGCAC	TGCGCGACGA
	8761		GACCTGGCGA		GGCCGACGGC	GTCACCCTGC	TGCGGAAACG
	8821	GTGACCGGGG	CGATGTCGGC	GGCGGTCAGC	GTCAGCGTCG	TOGGCGCGGG	CCTCGCGGAG
35	8881	GGCTCCAGAT	GCAGGCGTTC	GACGCCGGCG	GCGGAAGCGC	CCGCCACCTC	GGACACGCAG
	6941	GGGCAGTCGG	AGTCCGCGAA	GCCCGCGAAC	CGGTAGGCGA	TCTCCATCAT	GCGSTTGCGG
	9001	TOCGTACGCO	GGAAGTCCGC	CACCAGGTGC	GCCCCCGC30	GGGCGCCCTG	GTCCGTGAGC
	9061	CAGTTCAGGA	TOSTOGOAGO	GGCACCGAAC	GNCACGACCC	GGCAGGACGT	GGCGAGCAGT
	9121	TTCAGGTGCC	ACGTCGACGG	CTTCTTCTCC	AGCAGGATGA	TGCCGACGGC	GCCGTGCGGG
40	9181	CCGAAGCGGT	CGCCCATGGT	GACGACGAGG	ACCTCATGGG	CGGGATCGGT	GAGCACGCGC
	9241	GCAGGTCGGC	GTCGGAGTAG	TGCACGCCGG	TOGOGITAAT	CTGGCTGGTC	CGCAGCGTCA
	9301	GTTCCTCGAC	GCGGCTGAGT	TCCTCCTCCC	CCGCGGGT3C	GATCGTCATG	GAGAGGTCGA
	9361	GOGAGOGCAG	GAAGTCCTCG	TCGGGACCGG	AGTACGCCTC	CCGGGCCTGG	TOGOGOGOGA
	9421	AACCCGCCTG	GTACATCAGG	CGGCGCGGAC	GCGAGTCGAC	CGTGGACACC	GGCGGGCTGA
45	9481	ACTCCGGCAG	CGACAGGAGC	GTSGCCGCCT	GCTCGGCCGG	STAGCACCGC	ACCTCGGGCA
	9541	GGTGGAACGC	CACCTOGGCA	CGCTCGGCGG	GCTGGTC3TC	GATGAACGCG	ATCGTGGTCG
	9601	GTGCGAAGTT	CAGCTCCGTG	GCGATCTCGC	GGACGGACTG	CGACTTCGGC	CCCCATCCGA
	9661	TGCGGGCCAG	CACGAAGTAC	TCCGCCACAC	CGAGGCGTTC	CAGACGCTCC	CACGCGAGGT
						GTCGTCGAGC	
50						CAGCACGGTG	
	9841	AGGTGTTGTC	CAGGTCCCAG	ACCAGACACT	TGACAATGGT	CATGGCTGTC	CTCTCAAGCC
	9901	GGGAGCGCCA	GCGCGTGCTG	GGCCAGCATC	ACCCGGCACA	TCTCGCTGCT	GCCCTCGATG
	9961	ATCTCCATGA	GCTTGGCGTC	GCGGTACGCC	CGITCUACGA	CGTGTCCCTC	TUTUGUGUUT
	10021	GCCGACGCGA	GCACCTGTGC	GGCGG'I'CGCG	GCCCCGGCGG	CGGCTCGTTC	GGCGGCGACG
55	10081	TGCTTGGCCA	GGATCGTCGC	GGGCACCATC	T'CGGGGGAGC	CCTCGTCCCA	GTGGTCGCTG
	10141	GCGTACTCGC	ACACGCGGGC	CGCGATCTGC	1005066.00	ACAGGTCGGC	CCCCTCCCC
	10201	GCGACGAGTT	GGTGGTCGCC	GAGCGGCCGG	CCGAACTGCT	CCCGGGTCCG	GGCG!GGCC
	10261	ACCGCGGCGG	TGCGGCAGGC	CCGUAGGATC	CCGACGCAGC	CCCAGGCGAC	CGACITGCGC
60						CGCCGGAGCC	
60	10381	GUGCCGGCCG	GCACACGCAC	CTGGTCCAGG	TOUAGATUGG	CGTGGCCGGC	CACCACCACC
	10441	CUGGAUGGCT	TUGGGACGUG	CICGACGCGT	ACGUUUUUGG	TGTCGGCGGG	CHUGACCACC
	10501	ACCGCACCGG	AACCATCCTC	COCCEDENCE	AAGACGACCA ACACCCCCCC	GGTGGTCCGAG	CLCVVCCCCC
	10561	GUAGTCGTCC	AGACCITGIG	COMPOSICIONS	TCCCCCTCIC	CCCCGTCGAG TGAAGCCGAC	GGCCGCCACT
	10621	GTCCGCATCG	CCGACAGATC	661666666	16000LICAC	IGHMGCCGAC	10A0J0JJDD

	10001	TTCCCGCTGG	man comocime	aradi baama	CCCCCCTCTC	CGGCGTCGCC	CNCCCCCTCC
			CGGCCATGCC		ATGACACTGC	GCAGCGAACT	
	10801	COGACGISTS	CSSTGAACTC	GCCGTTCTCC	CGGCTGCCGA		GCCGTGCTCG
			COSCECAGAG	CAGGCCGTCG	GCGCCGAGCC	GGACGAGCAG	
5	10001		ADSTETCOOA	000000000	CGGTCACCGA	CAAGGTCGGT	
••′	10981			00036888888	GTGGACGAGT	GOGACCATGG	
		2000000000					TCTTCTCCAG
		Aldemna		GGT0003600 03AA0A603A	DOTGAGGCCC	COCTOCGOGA	
	11161		CAGTODGAGO		CTTGAGGAAC	GCGACCAACG	
10		0000T00T00	TTGACGGGTG	COGTCATGAG	AACACCTTCT	CGTATTCGTA	
1 ()	11281	cc3GTCTTC1	GGCCGTGGTG	TOCOTOGOGG	ACCITGCUGA	GCAGCAGGTC	
	11341		CGCCGGTGCG	TTTGTGCAGC	ACCCACAGOG	CGTCGACGAG	
	11461	CCGATCAGGT	CCGCGGTGCG	CAGOGGCCCG	GTCGGATGGC	CGAGGCACCC	
	11461		CCTCGACGGA	CSCGGTGCCC	TCCTGCACGA	TCCGCGCCGC	
15	11521	ATCGGGTGGA		COTGACGAAG	CCGGGGGGGGT	CCCGGACGAC	
. 5	11561		CCGCGAGCAG	STOCCOGGCG	GCGGCCATGG	CCTTCTCACC	
	11641		COTOGACOGT	CGGGATCAGG	TACGACGGGT	TCATGAAGTG	
			GCCGGGGCCAC	GGAGTCGGCC	AGTTCGTCAA	CCGGGATCGA	
	11761		GGATACCGGG	CSCCGCTGCC	GAGACCSTSS	CGAGTACCTC	
20	2 7 4 5 1	"CGGCGTCC"	CGACGACGGC	CTCGATCACC	GCGGTGGCCG		GGGCAGCGCG
	11991	GACGTGGCCG	TOOGCAGCAC	ACCGGGGTCG	GCCTCGGCGG	GCCCGGCCAC	
	11941	GTCCGCAGTT	CGGTGGCGAT	CCGCGCCCGC	GCCGCCGTAA	GGATCTCCTC	
		ACGAGTGTCA		GTGGCGCAGC	GCGAGCGTGG	TGATGCCGGT	
	12661	CCCGCGCCGA		CTGGTGGTCC		STECCTCCGG	
25	12121	GCAGCGAGTA			GGGTCGACCC	GATCGCGTCC	
	12181	GGCCGAGTTC		COGAGTT GCA	CGTCGAACGC	GATGTGGTCG	GCGAACGCGC
	12241	TGCCCGTCGA	GTOGAGGACG	CTCAGGCTGT	CCCGGTGGTC	CGCCGCGGTG	TCCGGTGCCG
	12301	CGCACAGGGC			CGCGGTCCGG	CAGTTGCTGG	TACTCGCCCT
	12361	CGGCGCGGGC	CTGCCCCGGA	TGGTCGACGC	AGATGAACGC	GTCGTCGAGC	AGGGTCTTCG
30	12421	GCAGTTCGGT	CTTGCCCGGC	TOGTOGGOGO	CGATGGCGTT	CACATGCAGG	TGCGGCAGCC
	12481	GCGGCTCGGC	GGGCAGCACC	GGCCCTTTGC	CCGAGGGCAC	CGAGGTGACG	GTGGACAGGA
	12541	CATCCGCGGC	GGCGGCGGCC	TCCGCCGGAT	CGGTCACCTT	GACCGGCAGT	CCGAGGAACG
	12601	CGATGCGGTC	CGCGAACGAC	GCCGCGTGGC	CGGGGTCGGT	GTCGCTGACC	AGGATCCGCT
	12661	CGATGGGCAG	GACCCTGCTG	AGCGCGTGCG	CCTGGGTCAC	CGCCTGTGCG	CCCGCGCCGA
35	10721	TCAGCGTGAG		TCGGACCGGG		GCTCGCGACG	GCGGCGACCG
	12781	CGCCGGTCCG	CATCGCGGTG	ATCACGCCTG	CGTCGGCGAG	GGCGGTCAGA	CTGCCGCTGT
	11841	CGTCGTCGAG	GOGOGACATO	GTGCCGACGA		CCGGAAGCGC	
	12901	GCGGACTGTA	CGAAACCGTC	TTCATGGTCA		GGGGACCCGG	
		ACTCGATGAC	GCCGGGAATG	TOGCOGCOGC		GGTACGCGGC	
40		CGAACTOSCO					
						GTCACGTTGG	
						CTTGGTGGTG	
						AAAATCTCGT	
						TOCOGCOGCO	
45	13331	CAGGGCGTCC	AGCCGGGTTC	CGATCGCGTC	CGCCTGGCGG	GCGCCCGGGT	CGACACCGGC
						GTCACCGGGT	
						GGGTAGTCGA	
						CGCAGCTGCA	
<i>5</i> 0						ATGTCCTCCG	
50	13621	GCCCAGGACG	GCCGCTGCCT	TCTGCCGGAC	GAGGGCGAGC	AGGTCGGTGG	GGCGTTCCTG
	13681	CTCGTTGCGG	GCGCTCCGGC	GGGCCGACGG	CTTGGGCCGG	CCACGCAGCA	GCGGGAGGTC
	13741	CGGCGGCAGG	TOGCCOGCCA	CGGCGACGAC	ACTGCCCGTT	CCGGTGTGGA	CGGCGGCGTC
	13801	GTACATGCGC	ATGCCCTGTT	CGGCGGTGAG	CGCGCTCGCC	CCACCCTTGC	GCATACGGCG
	13861	CCGGTCGGCG	TCGGTCAGGT	CCGCGGTCAG	GCCACTCGCC	TGGTCCCACA	GCCCCCALGC
55	13921	GATCGACAGC	CCTGGCAGCC	CTTGTGCACG	CCGGTGTTU	GCGAGCGCGT	CGAGGAACGC
	13981	GTTCGCCGCC	GCGTAGTTGC	CCTGACCGGG	GGTGCCCAGC	ACACCGGCCG	LCCGACGAGTA
	14041	GACGACGAAT	GCGGCGAGGT	CGGTGTCGCG	GGTGAGCCGG	TGCAGGTGCC	AGGCGGCGTC
	14101	GGCCTTGGGT	TTGAGGACGG	TGTCGATGCG	GTCGGGGGTG	AGGTTGTCGA	GCAGGGGGTC
(0	14161	GTCGAGGGTT	CCGGCGGTGT	GGAAGACGGC	GGTGAGGGGT	TGAGGGATGT	CCCCCCCCCCC
60	14221	GGTGGCGAGT	TGGTGGGGGT	CGCCGACGTC	GUAGGGGAGG	TGGGTGCCGG	CCCCCTCCTT
	14281	GGGGGGTGGG	GTGCGGGAGA	GGAGGTAGGT	GTGGGGGTGG	TTCAGGTGGC	CCACCCCCC
	14341	GCCGGCGAGG	GTGUUGGAGC	CGUCGGTGAT	GACGACGGCC	CCCTCGGGGT	CCAGCGGCCG
	14401	CGGGACCGTG	AGGAUGATCT	TGCCGGTGTG	CICGCCGCGG	CTCATGGTCG	CCAGCGCCIC
	14461	GUGGACCTGC	UGCATGTCGT	GUACCUTUAC	(Judijakija)	TGCAGCACAC	CUCUCUCUAA

	14521	CAGGCCGAGC	roomgoooda	772777777	GAGCCGGTCG	COCCCCCC	CCATCACCTC
	14581		TGGACGGGGT		-03TCTT0000	ATOTOGATCA	
	14643	0AA0331030 0300000A30	AGGCGGACGG	ACROSTOSAS	PASTTCACCS	STGAGCGAGT	TGAGCACGAC
	14701	GTCGACCGGC	GGGAACĞCGT	CGGCGAACGC	agreerses	SAATOGGCCA	GATGCGCTCC
5	11761	GICCAGGICC	ACCAGATGGC	6077030630	GCTGGTGGTC	GOGTACACCT	CCGCGCCCAG
	14821	31000000000	ATCTGCCGGG	0000005550	SACACEGEES	DIGGCCGCGT	GGATCAGGAC
	14581	0770703000	GGGCGCAGCC	00000A00T1	GACCAGGCAG	TACCACGOGG	TOGOGAACGO
	14341	GGTTATTATG	SACGCCGCCT	30000AN007	2280003702	330AT003G0	CGAGCATCCG
	15001	3733773303	ATGACCGTGG	G3003AA300	3373203M37		CGCGGTCGCC
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	15121	GAGGAGGGCC	TGACCGGGGT	AGGTGCCGAG	COCCATCACC		AGTTGAGGCC
	15181	CGCCGCACGC	ACACCGATCC	GGACCTCGGC	CGGGGCGAGG	J3G0G0CGGG	GCTCCGCCGA
	15341	GTCGGCCGCG	GTGAGGCCGT	CGAGGGTGCC	CGTCCGCGTC	SGCCGGATCA	GCCACGTGTC
	15301	COTGTCCGGC	ACGGTGAGCG	GCTCCGGCAC	CCGGGTGAGG	CGGGCCGCCT	CGAACCGGCC
15	15361	GCCGCGCAGC	CGCAGACGCG	GCTCGCCGAG	TGCGACGGCG		GCTCGGGGGC
	15421	GAGCGTGACG	COGGACTOGS	TCTCGACGTG	GACGNACCGG	COGGGCTGCT	CGGCCTGGGC
	15481	GGGGGGGAGG	AGTECGGCCG	CCGCGCCGGT	GGCGAGCCCC		GCACGAGCAG
	.6541	Amogeoedee	GAGCCGGTCA	GGGCGGTCI	CAGCCGGGTG	STGAGCGCAC	GCGTCTCGGC
	1560:	CACCGGGTCG	TOGOCATOAG	CGGCAGGLLA	CGTGATGACG	TUCACGTCGG	TCGCGGGGAC
20	15861	ATCCCTGGGT	GCGGCGACCT	CGATCCAGGT	GAGACGCATC	AGGCCGGTGC	CGACGGGTGG
	15721	GGACAGCGGG	CGGGTGCGGA	COSTCOGGAT	CTCGGCGACG	AGTIGGCCGG	CGGAGTCGGC
	15781	GACGCGCAGA		CGCCGTCACG	AGTGATCACS	GCTCGGAGCA	TGGCCGAGCC
	15841		AACCGGGCCC	CCTTCCAGGC	SAACGGCAGA	COCGCAGOGO	TGTCGTCCGG
	15901	CSTGGTGAGG	GCGACGGCGT	GCAGGGCCGC	GTCGAGCAGD	SCCGGATGCA	CACCGAAACC
25	15961	andegeereg	GCGGCCTGCT	CGTCGGGCAG	COCCACCTOS	SCATACACGG	TGTCACCATC
	16021	ACGCCAGGCA	GCCCGCAACC	CCTGGAACGC	CGACCCGTAC	TOATAACOGG	CATCCCGCAG
	16081		AACCCCGAGA	OGTOGACGGO	CACGGCCGTG	ACCGCCGCC	ACTGCGAGAA
	16141	COGCTCCACA	CCGACAACAC	COSCOGTETO	GGGGGTGTCG	GGGGTCAGGG	TGCCGCTGGC
	16201	GTGCCGGGTC	CAGCIGCCCG	TGCCCTCGGT	ACGCGCGTGG	ACGGTCACCG	GCCGCCGTCC
30	16261	GGCCTCATCA	GCCCCTTCCA	CGGTCACCGA	CACATCCACC	GCTGCGGTCA	CCGGCACCAU
	16321	AAGGGGGGAT	TCGATGACCA	GCTCGTCCAC	TATCCCGCAA	CCGGTCTCGT	CACCGGCCCG
	16381	GATGACCAGC	TCCACAAACG	CCGTACCCGG	CAGCAGGACC	GTGCCCCGCA	CCGCGTGATC
	16441	AGCCAGCCAG	GGGTGAGTGC	GCAATGAGAT	CCGGCCAGTG	AGAACAACAC	CACCATCGTC
	16501	GGCGGGCAGC	GCTGTGACAG	CGGCCAGCAT	CGGATGCGCC	GCACCCGTCA	ACCCCGCCGC
35	16561	CGACAGATCG	GTGGCACCGG	CCGCCTCCAG	CCAGTACCGC	CTGTGCTCGA	ACGCGTACGT
	16621	GGGCAGATCC	AGCAGCCGTC	CCGGCACCGG	TTCGACCACC	STGTCCCAGT	CCACTGCCGT
	16681	GCCCAGGGTC	CACGCCTGCG	CCAACGCCGT	CAGCCACCGC	TOCCAGCOGC	CGTCACCGGT
	16741	CCGCAACGAC			CATCGCCGGC	AGCAGCACCG	GATGGGCACT
	16801	GCACTCCACG	AACACCGACC	CATCCAGCTC	CGCCACCGCC	GCGTCCAACG	CCACCGGACG
40	16861		CGGTACCAGT			GTCACCCAGG	
	16921				CCCTGCCACC		
					GTGGGAGGCG		
	17041	CACCCGCACG	CCTTCGGCCT	CATACCGCGC	CACCACCTCC	TCCACCGCCG	ACGGGTCCCC
					CGCCGCGATC		
45					CATCGCTCCC		
					GGCGGCGTCC		
					GTGTCCGATC		
					CGCGACCGCC		
					CAACATCTCC		
50					ACGCGCGGCG		
					GCCCTGGCCG		
					GGCATCGCCC		
					CGCGACCGCG		
	17701	GCGCAGATAC	CCCTCCAGCC	GCTCCACCTG	CCCCCGCAGA	CTCACCTCAC	CACGAGCCGA
55					CGACTCCCCA		
					CACCCCGAAC		
					CTCGGTGAGC		
					ATGCAGCGTC		
					GACACCCCC		
60					AACCTCACGC		
	18121	AATGGCCTGC	GCCTCGATGG	GATCGCCCAG	CGTCGTCCCC	GTCCCGTGCG	CCTCCACCAC
					CACCAACGCC		
					GGAGGCACCG		
	18301	GCGGACGACC	GCGAGAACGG	TGTGTCCGTT	GCGCTCGGCG	TCGGAGAGCC	GCTCCAGCAC

	1000	AAGAACGCCG	0000000000	202122222	2020882000	000000000	2222222
		GCGGCCGTCG					
		CATGACGGTG					
		GGCCTGGTGC					
5		GTGGAGCCCA				CTGGGGCTGCA	
	18661	GCCGAACCCG				GAGAAGGCGC	
		GCCGGTGTCG					
	18781	TGTCGTTTCC		GCTGCTGGGG			GGGGGGTGAT
	18841			A0000000000		AAGCCGCCGC	· · · · · · - · · · · · ·
10		CGATCCGCCG				GCCGGGAAGC	
	16961	GTCGCCGCCA	CTGTCCACCA	TGCGCACAG	spagrassa		
		TOGGCAGGCC	Angedeadga	TOGOCARCAS	TTCGTCACGG	GTCGCGGCGG	OTSTGGGAAC
	19081	AGGGACCGGT	GCGGCACCAC	CGACCAGAGO	CTCGTCCAAC	CGCGACGCGA	TGGCCCGCGG
	19141	CGTCGGGTAG					
15		GTTCCGCAGT					
		GGACACGTCC					
		CAGCAGCGCG					
		ggcggtggcc					
	19441	STGCGCGGTG	AGGTCCATCG	TGGCCGCCAC	GGCGAACGC	STGCCGGTTC	dggddridggd
20	19501	TTCCAGCAGG	CGCATGCCCA	CACCGGGGGA	CATGGGGGGGG	AAACCGCCGC	GGCGGACACG
	19561	GGTGCGGTTG	GTGCCGCTCA	TGCTGCCGGT	GAGTCCCCTC	TCATCGGCCC	AGAGGCCCCA
	19621	GGCCAGCGAC	AGCGCGGGCA	GTCCTTCGGC	ATGGCGCAGC	GTCGCGAGTC	CGTCGAGGAA
	19681	CCCGTTCGCC	GCCGAGTAGT	TGCCCTGGCC	GCGGCCGCCC	ATGATGCCCG	CBACGGACGA
	19741	GTAGAGGACG	AACGAGCGCA	GGTCCGCGTC	CCGGGTCAGC	TOGTGOAGGT	GCCAGGCGCC
25		GTCGGCTTTG					
		GTCGTCGAGC					
		CGCGGCGGCG					
		CGCCGGCGGT					
		ATGCCGGGCG					
30		CGGGTCGAGC					
		GTACCGGCCG					
		CTCGATGGGG				CGGCCCGGGT	
		GGCGGACCGG					
25		GAGGGTGGTC					
35		CTCGGTGAGC					
		GATGTGGACC GTACAAGGAG					
		CGCGGCGACG					
	20561					GTCGTGTGGA	
40		GCTCCACGAG					
40		GACGTGCAAG					
		CTGTTCCCCG					
		CAGTCCCTGG					
	20941	GCTCACGTCG	ACGCGTCGCG	CGCCCGGCGG	CGGCCACGCG	GGCGGCGGGA	COGCOGCGAC
45		GCTTCCGGCC					
		ACGCGCGTGG					
		CACATCCACC					
		CACCCCGCAA					
		CAGCAGAACC					
50		CCGGCCAGTG					
		CATCGGATGC					
	21421	CAGCCAGTAC	CGCCTGTGCT	CGAACGCGTA	GGTGGGCAGA	TCGAGCAGCC	GTCCCGGCAC
		CGGTTCGACC					
		CGTCAGCCAC					
55		TTCCATCGCC					
		CTCCGCCACC					
		ATCCACCGGC					
	21781	CCCGCCGGAA	ATCCCCTCCA	GTACCTCGGC	CAACTCGTCC	TCGATGGCTT	CCACGTGGGG
		CGTGTGGGAG					
60		CGTCACCACT					
		ACGCGCCGCG					
	22021	AGCCATCGCC	CCCCGCCCGG	CCAGCCGCCC	GGCGATCACC	TGGCTGCGCA	AGGCCACCAC
	22081	GCGGGCGGCG	TCCTCAAGGC	TGAGGGCTCC	GGCCACACAC	mccca ca ccc	TOTOGCCCCC
	22141	GGAGTGTCCG	ACCACCGCGT	CCGGCACGAC	CCCATGCGCC	TUCCACAGCG	COGCCAGGCT

				22222002222		222222222	CA WOOCCOOC
	22201	CACCGCGACC	GCCCAGCTGG	COGGCTGGAC		CGCTCCGCCA	
		COCCACATU	TOCCOCAUAN		GTGCGGCAAC		CACACTCCTC
		CATATGAGGG	SOGRACAUUS	omantimbed	CAICAACICO CAASAAAAA		CCACCCACTG
5	02381	ALCAUCUTSC	00000000000000000000000000000000000000	CONTRACTOR OF CO	MUDUCACION		CACCCATCAC GCACCAACCC
7,	22441	CCGGGGGATCG	CCCAACAACA	COGCACGGTA	ACTION OF THE		GCCGCTCCAC
	2000	0.000000000	SCHOOLOGE	CLAUNCUAUC OF ATRACT CO.	occasianam		ACCCATCGAC
	0.0001	r 000010000	AGACTCACCT	CACCOCCAIC	LONGONGER		CGTTCGTACC
	22621	AGCCGACIUU	COACGOGACG	ar caccacan	ACCOUNTERNAME ACCOUNT		GCCACGCCCG
10	225 <u>91</u> 22741	DO FURDICUO .	AAAGCGGAGA	CACCGGCCCC	GCGCGGACGT		ACGGCTCGTC
10	22801	CSCCTCGSTS	AGCAGTTCCA		HOCOLDON	ATGACCATCT	TGATGACACC
		CACALAGAA	GTCTTCGGCG	CONTOCCATA		TTCAACGAAC	CCAGCAGCAG
	22861	GGCGACACCC	GCAGCCGCCT CGCTCCTGCC	CGTACGTCGC	om.b.llugno omenmones	TGCGCCTCGA	TGGGATCGCC
	92921 22981	CAGCGTCGTC	accemences.	00000000000	CACGTCCACG		CGAGCCUCGC
15	23041	CTTGTGGAGG	GCCTGGCGGA	TGACGCGCTG	CTGGGAGGGG		CGGAGATGCC
13	23041	STTCGAGGCG	CCGTCCTGGT	TGACGGCGGA	CALARAGERS	ACCGCGAGGA	CGGTGTGTCC
	23161	31.00N0000	GCGTCGGAGA	GCTTTTCGAC	SACGAGGAGA	CCGGGCCCCCT	CGGCGAAACC
	00000		GCCGCGTCAG	CGRACGCGTT			CGCCGCCC1
	23281	0.000010110	TOCACGRAGG	TOTOTOTOTOA	TGCCATCACT	GTGACACCAC	CGACCAGCGC
20	23341	CAGCGAGAAC	TOCCOGGTCC	GCAGCGCCTG	CCCGGCCTGG	TGCAGCGCGA	CCAGCGACGA
20	23401	TGAACACGCC	GTGTCGACCG	TGACCGCCGG	ACCCTCCATG	COGAAGAAGT	ACGACAGCCG
	23461	TOOGGOGAGO	ACCGCGGGCT	GTGTGCTGTA	GGCGCCGART	CCGCCCAGGT	CCGCGCCCGT
	23521	COCCUTACOO	TAGTAGAAGC	CGCCGACGAA	GACGCCGGTG	TOGOTGOOGO	GCAGGGTGTC
	23581	CGGCACGATG	CCGGCGTGTT	CGAGCGCCTC	CCAGGCGATT	TOGAGGAGGA	TOOGOTGOTG
25	23641	CGGGTCGAGT	GCGGTGGCCT	CGCGCGGACT	GATGCCGAAG	AMCGCGGCAT	CGAAGTCGGC
	23701		AGTGCGCCGG	CCCGCCCGGT	GGCGGACTCG	GCGGCGGCGT	GCAGCGCGGC
	23761	CACGTCCCAG	CCGCGGTCGG	TGGGGAAGTC	GCCGATCGCG	TOGOGGGGGGT	CCGCGACGAG
	23821	CTGCCACAGC	TCTTCCGGTG		GCCCGGCAGT	CGGCAGGCCA	TGCCGACGAC
	23881	GGCGAGCGGC	TCGTTCGCCG	CGGCGCGCAG	CGCGGTGTTC	TCCCGGCGGA	GCTGCGCGTT
30	23941	GTCCTTGACC	GACGTCCGCA	GCGCCTCGAT	CAGGTCGTTC	TOGGCCATCG	CCTCATCCCT
	24001	TCAGCACGTG	CGCGATGAGC	GCGTCTGCGT	CCATGTCGTC	GAACAGTTCG	TCGTCCGGCT
	24061	CCGCGGTCGT	GGTGCTCGCG	GGTGCCTGTG	CCGGTGGTTC	ACCGCCGTCC	GGGGTCCCGT
	24121	TGTCGTCCGG	GGTCCCGTTG	ACGTCCGGGG	CCAGGAGGGT	CAGCAGATGA	CGGGTGAGCG
	24181	CGCCGGCGGC	GGGATAGTCG	AAGACGAGCG	TGGCCGGCAG	CGGAATGCCG	AGGGCCTCGG
35	24241	AGACCCGGTT	GCGCAGGCCG	AGCGCGGTGA	GCGAGTCGAC		TTGAACGCCG
	24301	TGGTGGCCGT	GACCGCCGCC	GCGTCGGTGT	GGCCCAGCAG	GGTGGCGGCG	GTGTCGCGGA
	24361	CGACGCCGAG	CAGCACCTGT	TCCCGTTCCT	TGTGGGGGCAG	GTCCGGCAGG	CGTTCCAGCA
	24421	GGGAGCCGCC	GTCGGTCGCG	GAGCGCCGGG	TGGGGGGGCTG		CACAGCGGTG
	24481	ACGGGTCGCC	GGGCCCGGGT	GGGGCGGTCG	CCACGACCAC		GTGGCGCACG
40	24541	CGGCGTCGAG	GAGGTCGGTC	AGCCGGTCCG	CCGCGGCGGT	GAACGCCACG	
	24601	CTTGTGCCCG	GCGCAGGTCG	GCCAGGGCCT	GSAGCGGTCG	GGCCGCCTCG	CCGGACGGAA
	24661	CGGCGAGAAC	GAACGCGGTC	AGGTCGAGGT	CGCGGGTCAG	GCGGTGCAGT	TCCCAGGCCG
	24721	ACTCGGCGGT	GCCGTCCGCG	TGGACGACCG	CGGTCACCGG	GGTTTCCGGC	ACTGTGCCCG
	24781	GCTCGTACCG	GATCACTTCG	GCGCCGTGTC	CGCCGAGGTG	TOOGGCGAGT	TCCTCCGAAC
45	24841	CGCCCGCGAG	GAGGACGGTG	TOGOCGTACG	AGGCCGCGGC	CGTGGTGGGC	GCGGCGGGGA
	24901	CGAGGCGGGG	CGCTTCGAGG	CGCCCGTCGG	CCAGGCGCAG	GTGCGGTTCG	TCGAGGCGGG
	24961	AGAGGGCGGC	GGCGCGGCGG	GGGGTGACCG	TGTCGGTGGT	CTCCACGAGC	ACGAGCCGGC
	25021	CCGGTTCCGC	GGTGTCGAGC	AGTGCGGCGA	CGGCACCGGC	GACGGGCCCG	GCCTCGGCGG
	25081	ACACCACCAG	CGTGGCGCCG	GOSTTOCTOR	GATEGTECAG	TGCGGTACGG	ACCTCGTCGG
50	25141	GACCGGATAC	CGGGACGACG	ATGACGTCGG	GCGTGGCGTC	GTCGCCGAGG	TCGGTGTACC
	25201	GGGGGGCCGT	GGTGCCGGGT	GCCGCCGGGG	CCCGGACGCC	GGT CUAGGTG	CGCCGGAACA
	25261	GCCGCACGTC	CCCGTCCGGG	CCCGTCGTGG	CGGGGGGGGG	GGTGATGAGC	### TAGEOGE TOTAL
	25321	GAGCCACCGG	CCGTCCCAGT	TCGTCGGCGA	GGTGCACGCG	GGCGCCGCCC	n coccommen
	25381	CGTGGACGAA	GGTGACGCGC	AGTTTCGTGG	CGCCGCTGGT	GTGGACACGG	ACGCCCTTCCA
55	25441	ACGCGAACGG	CAACCGTACC	CCCGCGTTCT	CGGCGGCCGC	COCCATGOTO	CCCGCTTGCA
	25501	GCGCGGTGAC	GAGCAGCGCC	GGGTGCAGTG	TGTAGCGGGC	GGTGCCCTG	CACARCCCCC
	25561	CGTCGAGGGC	GACTTCGGCG	CAGACGGTGT	CTCCGTGGCT	DUDDUDAUU	GACATGCCGC
	25621	GGAACTCGGG	GCCGAACTCG	TATCCCGCGT	CGTCGAGTCG	CTGGTAGAAG	TO COMPOSE
	25681	CGACCGGTTC	CGCGTGCTCG	GGCGGCCAGG	GCCCCGGCGT	GETGGCCGGT	CTCCCCCCC
60	25741	CGATGCCGGC	GAAGCCGGAG	GCGTGGCGGG	TOCATGTTTT	GREGORGAGE	CCCACCECCA
	25801	GGACGCGCAC	GGCACGGCGT	CCGGTGTCGT		GAUGUTUAUG	DCCACCTCCC
	25861	CGGCGCCGGT	GGCGGGCAGG	ACCAGCGGTG	TOTOGAUGAC	CAGITICGICG	CCCACCACTA
	25921	AGCCTGCCTC	GTCGGCGCCG	CGTCCGGCCA	. ALIUCAGGAA	CCCCACCCAC	A A C C C C C C C C C C C C C C C C C C
	25981	CGGCGCCGTC	GACGGAGTGA	CUGGCCAGCC	A FOURTURE T	GGCCAGCGAG	AACCOOCCOG

				M40000-			
		TGAGCAGCAC				GGCGAGCAGC	
	26104	COGCOTCGAG	TOOGAGGCCG		TGCCGGCCGC	GGTCTCGATC	
	261.61	CATGGTGGAA	GGCGTATGTG	GGCAGGTCGT		CGTCGCGGGG	ACGACCGCCG
~	2,62,21	CCCAGTCGAC	GGGCACGCGG	STTSTGTGCG	CCTCGGCCAG	CGCGGTGAGC	AGCCGGTGGA
5	0.6061	37000000000	GCGGCGGAGC	STGGCGACGG	TCGCSSSSTC	GATOGOGGGO	AGCAGCACGG
	36341	GGTGCGCGCT	GACCTCGACG	AACACGGTGT	CRCCCGGCTC	GOGGGCAGCG	GTCACGGCCG
	26401	TBBCGAAGCC	TACGGGGTGG	CGCATGTTGC	GGMCCMGTA	CTCGTCGTCG	AGCGGCGCGT
	26461	CGATCCAGCG	TTCGTCGGCG	GTGGAGAACC	ACGGGATGIC	GGGCGTGCGC	GAGGTGGTGT
	16531	COGCGACGAT	CCGCTGGAGT	TOSTOGTACA	900000129AC	GAACGGGGTG	TGGGTCGGGC
10	26581	ASTOGACGGO	GATGCGGCGC	ACCCAGACGC	0303330070	GTAGTCGGCG	ATCAGCGTTT
	26641	CGACGGGGGTC	CGGGGGGGGG	GOGACGETCC	1331331333	GCCGTTGCGG	CCCGCGACCC
	26701	AGACGCCGTC	GATCCGGGCG	SCATOCOCCT	30A30.0030	GGCCGGGAGC	GCGACCGAGC
	26761	COATEGOGGG	GCSTCCGGCG	AGTTCGCGCA	33A 11432A3		AGCGCGACGA
	26821	GGCGGGCACC	GTCCTCCAGG	GTGAGCGCTC	COSCONDACA		ATCTCGUCCT
15	26881	GGGAGTGTCC	GATGACGGCG	TOOGGGCGTA	0300030860		GCGGCCAGCG
• •	26941	ACACCATGAC	GGCCCAGCAG	ACGGGGTGCA	CGACGTCGAC		ACCTCCGGGT
	27001	CGTCGAGCAT	GGCGATGGGG	TOCCAGCOCS	TOTOCHOGAT	CAGCGCGTCG	GCGCATTGGC
	27061	GCATCCTGGC	GGCGAACACC	GGGGAGGCCG		GAGGGCGATG	CCGCGCCACT
	0001	30000000000			- COARDON COM		
20		00100100110	TOTGGGGAAG	ACGAAGACGG		GGTGAGCGCC	GTGCCGGTGA
20		CGACGTCGTC	GTCGAGCAGC	ACGGCGCGGT	GCGGGAAGGT	CSTACGCCTG	GCGAGCAGGC
	2/24:	CCGCGGCGAT	GGCGCGCGG	TOGTGGCCGG	9A00960930	GAGGTGCTCG	CGGAGTCGGC
	27301	GGACCTGGCC	GTCGAGGGCC	GTGGCGGTCC	GCGCCGAGAC	GBGCAGTGGT	GTGAGCGGCG
	27361	TGGCGATCAG	CGGCTCACCG	GGCTTCGAGG	CCCACCACT	CTCGGCCGGC	GGCTCCCCGG
2.5	27421	0033676660		ACGTGGGGGT	regradaser	GACGCCGAAG	GAGGACACAC
25	27481	CGGCGCGCCG	CGGGCGGTCG	GTCTCGGGGCC	AGS30033330		AGTTCGACGG
	27541	CGCCGGCCGT	CCAGTCGACG	TGCGAGGACG	GOGTOTOCAC	GTGCAGGGTG	CGCGGCAGGG
	27601	TGCCGTGCCG		ACCATCTTGA	TGACACCGGC	GACACCCGCG	GCGGCCTGAG
	27661	TSTGGCCGAT	GTTGGACTTC	AGCGAGCCCA	GCAGCACCGG	GGTGTCGCGC	CCCTGCCCGT
•	27721	AGGTGGCCAG	CACCGCCTGT	GCCTCGATGG		CCTGGTGCCG	GTGCCGTGCG
30	27781	CCTCCACGGC	GTCCACGTCC	GCCGGGGTGA	GCCCGGGGGTT	GGCCAGGGCC	TGCCGGATCA
	27841	CCCGCTCCTG	CGAGGGCCCG	TTCGGCGCCC	ROARCOCOTT	GGAAGCACCG	TCCTGGTTGA
	27901	CCGCCGAACC	CCGGACAACC	GCCAGCACAC	GGTGGCCGTT	GCGCTCGGCA	TOGGAGAGCO
	27961	TCTCGACGAT	CAGCACACCG	GACCCCTCGG	CGAAACCGGT	GCCGTCAGCC	GCATCCJCGA
	28021	ACGCCTTGCA		GGCGCGAGAC	CCCGCTGCTG	GGAGAACTCG	ACGAAGCCGG
35	28081	ACGCCGAGGC	CATCACCGTG		CCAGGGGGAG	CGAGCATTCG	CCGGAGCGCA
	28141	GTGACTGCCC	GGCCTGGTGC	AGCGCCACCA	GOGACGACGA	ACACGCCGTG	TCGACCGTGA
	28201	CCGCCGGACC	CTCCAGACCG	TAGAAGTACG	ACAGCCGACC	GGACAGCACA	CTGGTCTGGG
	28261	TGCCGGTCGC	GCCGAAACCG	CCCAGGTCGG	TGCCGAGTCC	GTACCCGTCG	GAGAAGGCGC
	26321	COATGAACAC	GCCGGTGTCG	CTTCCGCGCA	GCGACTCCGG	GAGGATCCCG	GCGTGTTCCA
40	28341		CGAGGTCTCC			GTCCATCGCC	
	26441	GCGGACTGAT	CCCGAAGAAC	GCCGCGTCGA	AGTOCGCCAC	CCCGGCGAGG	AAGCCACCAT
	28501	GACGCACGGT	CGACGTGCCC	GGATGATCCG	GATCGGGATC	GTACAGCCCG	TOCACGTCCC
	28561	AACCACGGTC	CGTCGGAAAC	GCCGTGATCC	CGTCACCACC	CGACTCCAGC	AGCCGCCACA
	28621	AGTOCTCCGG	CGACGCGACC	CCACCGGGGA	GCCGGCAGGC	CATCCCCACG	ATCGCCAACG
45		SCTCGTCCTG					
	28741	GCGCCGCGGT	GAGCTTCGCC	GOGACGGCGC	GCGGCGTCGG	GAAGTCGAAG	ACCGCGGTGG
		CGGGCAGCCG					
		AGTCGACGCC					
		CGAGTACGGC					
50		CGGAGAGCCG					
		CCCGGCGCGG					
		GCGCCGGGTC					
		GCGCCGTCAC					
		GTTCCCACAG					
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		CACCGGCGGC					
		GCAGGTGCCA					
		GCGCGGTGAG					
		GCGCCGGGTC					
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00		GCAGCCGGCG					
		CGGAGCCACC					
		GGACCGCCGG					
		CATCGAGCGC					
	29021	CHICGAGCGC	GG LGGCCGCT	CUBAGCAGCG	9C1C06C661	97666666	GCGICGHCGH

	00001			maaaaaa	220		
		GGACGATCCG			CGGTCCGCAC	CAGTCCGGCG	
		ACGCGAGACC	GGGCCCGGTG	TGGACGGCCA	GGACCGCGTC	GGCGTACCGG	TCGTCGGTGA
	30000	GUSCOSAAGE	DACGGGGGTC	AGGACGCCGG	CGCCCAGTTC		TOGAGOGGGG
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5	30121	GGCCGGTCGT	0000000000	GGCGGCAGCT	COGGGRACTIC	BBCGAGCACC	GGGCGCAGCA
	30181	GGCCCGGAAC	GGGTCCCGTS	ACCGTCAGGG	GGGGGCCTGCG	Chassesses	ATGGTGGCGA
	30241	0999000000	GGTCTCGTCC	GCGAGGTGTA	ccccccccac	GGTGACGGCG	ACGCGTACCG
	30301	COGTGGCGCC	SGTGGCGTGG	ACGCGGACGT	COTOGRAPOSO	GTACGGAAGG	TGGTCCCCTT
	30361	CCGCGGCGAG	GCGGAGTGCG	GCGCCGAGCA	GCGCCGGGTG	CAGGCCGTAC	CSTCCGGCGT
10	30401	EGGCGAGCTG	TOOGTOGGOG	AGGGCCACTT	COGCCCAGAC	GSCGTCGTCG	TOGGCCCAGA
	30481	dagagagag	GCGGGGCAGC	goggggccggT	COGTGTAGGG	SACTOGGGCC	AGACGGTCGG
	30541	COATSTOGES	GGGGTCCACC	93000000000	7000000733		GGCATCTCCC
	33601	30A0000033		GGGTCGGGGG		GTGCGCGTGC	TOGGTCCACT
	30661	0000000000	GTGCCGCGTG	T(C10 33731	0000000000	GCCGTCCGCC	
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1.5	30781	TGAACGTGTC	GAGGGCGCCG	CAGCCGGCTT	OGTOGOCCOC		AGATCCAGGA
	30641	GGGCCGCGGC		GCGAGGGGGT	GCAGGGAGTG		TOGGCGGCGT
					TGCCGGGCAG		
	30901	CGACCCGGCC	GGTGAGCACC	AGGTCGGCGG		GGTGACCGCC	GOGGTCAGCG
20	30961	CCGGGTGCGC		TGTCCGGCCG	GGGCCGCGTC	GCCCGCGGTC	TGGGTGCCGA
20	31021	GCCAGTAGCG		AACGGGTACG	TOGGCGGGTG	CGAGGCGCGT	GCCGGCGCGG
	31081		CTTCGGCCAG	TOGACOGTGA	CGCCGTCGGT	GTGCAGCCGG	GCGAGCGCGG
	31141	TCAGGGGGGA		TOGTOGGOGT	GCAGCATOBB		ACGAGTCGGG
	31201	TCAGGCTCCG		ATCTCCAGGA	SCACOGGGGG	GTGGTGGGGG	GCGACCTGTT
	31261	CCCCGAACCG	GACGGTGTCG	CGGACCTGTC	GCACCCASTA		GTGCAGGCGG
25	31321	CGCCCGCGGC	CATCGGGATC	CTCGGCTCGT			ACCTTGCGGA
	31381	ACTCCTCGAG	CATCGGCTCC	ATCCGCGCCCG	AGTGGAACGC	GTGGCTGGTC	CGUAGGCGGG
	31441			GCGACGTCGA	GCACCGCCTC	CTCGTCACCG	GAGAGCACGA
	31501		CCCGTTGACC	GCGGCGATCT		CCGCAGCAGC	GGCAGCGCGT
		CCCGTTCCGA		GCGGCCATCG	CCCCGCCGGA	CGGCAGCGCC	TGCATCAGGC
30		GGGCCCGTGC			CCTCCAGGGA		GCGACGTACG
	51681	CGGCGGCCAG	CTCGCCGATC	GAATGGCCCA	CGMAGGCGTU	CGGGCGTACG	CCCCACGCCT
	31741	CGAGCTGTGC	GCCGAGTGCG	ACCTGGAGCG	CGAACACCGC	GGGCTGGGCG	TACCOGGTGT
	31801	CGTGGAGGTC	GAGCCCGGCG	GGCACGTCGA	GGGCGTCCAG	CACCTCGCGG	CGAGTGCGCG
	31961	CGAAGACGTC	GTAGGCGGCG	GCCAGTCCGT		GGGACGTTGT	GAGCCC1'GTC
35	31921	CGGAGAAGAG	CCACACGAGG	CGGCGGTCCG	GTTCTGCGGC	GCCGGTGACC	GTGTCGGTGC
	31981	CGATCAGCGC	GGCCCGGTGC	GGGAAGGCCG	TGCGGGCGAG	CAGGGCCGCG	GCCACCGCGC
	32041	GCTCGTCCTC	CTCGCCGGTG	GCGAGGTGGG	CGCGCAGGCG	GTGTACCIGT	GCGTCGAGTG
	32101	COTGOGGGGT	GCGTGCCGAG	AGCAGCAGGG	GCAGCGGTCC	GGTGTCGGGT	GCCGGGGCGG
	32161	GTTCGGGGGG	CGGTCGGGGG	TGGCTTTCGA	GGATGATGT3	AGCGTTGGTG	COGCTAACGC
40	32221	CGAAGGAGGA	CACCCCGGCG	CGCCGTGGGC	GGTCGGTTTC	GGGCCAGGGG	
	32281	TGAGGAGTTC	GACGGCGCCG	GCCGTCCAGT	CGACGTGCGA	GGACGGCGTG	TOCACGTGCA
	32341	GGGTGCGCGG	CAGGGTGCCG	TGCCGCATGG	CGAGGACCAT	CTTGATGACA	COGGOGACGC
		CCGCGGCGGC					
		CGCGATGCTG					
45		TCCCGGTGCC					
		GCGCCTGCCG					
		CACCGTCCTG					
	32701	CGGCGTCGGA	GAGCCTCTCG	ACGATCAGCA	CATCOCONEC	CTCGGCGAAA	CCGGTGCCAT
	30221	CAUCCGCATC	CCCCAACCCC	TTCCACCGGC	carcoccas	enegeocorari	TOCTOGGAGA
50		AGTCCACGAA					
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	22001	COGTGTCCAC	COTTOTOTOTO	100000001	77CCC 7677	CTLCCLCLCC	COLCOGRACE
	22001	GCACACTGGT	COTGACCGCC	CHCCCTCCA	P N CCCCTACAT	OTCOCCCCA	GTGCCG (ACC
	33031	CGTAGAAGTA	CIGGGIGCIG	TI CACCCCCA	TOTOCOTTOO	GCCCACCGEC	TOCGGGAGGA
55	22707	TCCCGGCGTG	TTT COLC COLC COLC	MACACGCCGG	TGTCGCTTCC	CACACCCTCC	TOCOGGGTCCA
ננ		TCGCCAGCGC					
		CGAGGAAGCC					
		GCCCGTCCAC					
60		CCAGCAGCCG					
60	33421	CCACGATCGC	CAACGGCTCG	AUGUUGU A	COCCCCCCC	COGGGGTAUGU	CHCCCCTCCT
		TGGCCCGCGC					
	33541	CGAAGACGAG	CGTAGCGGGC	AGCGTCAGGC	CCGTCGCGTC	COCCOCCCC	COCARCOCO
	33601	CGACGCCGGT	CAGCGAGTCG	AAGCCCACTT	CCUTGAACGC	CARCARCE CARCA	BCGAIGGCGT
	33661	GGGCGTCGCG	GTGGCCGAGC	AUCGUGGCAG	CGCTGGTACG	GACGAGGTCG	AGCATGTCGC

	33721	GCGCGGCCGG	ngamagania	gmg0g0cca:	gaaaagaaa	alacaracan	AGGACCGGCG
	33781	GGACCCGGTC		ACGGCGGCGA		GATCGGCACG	
	338 11	SGTCGGTGTG		TOGRACAGGG		TGCGGCCGTC	
	33901		GGCGATGCGG		TBBCGCTCAG	CCGCCCGCCC	
5	33961			GCGAGCGAGA			TGCCGGTGGC
		00000000000000000000000000000000000000	GTOGAGGAAC		TOGOGTAGTT	GGCCTGACCC	GCGCCGCCGA
	34081	ACCTGCCGA		TACAGGACGA	Accondication	GIOGAGATOG	
	34141	CGTGCAGGTG	CCAGGCGACG	TOCGCCTTGA	deegewaaw	BECETCOCAC	
	34201	GOATGGTCGT	CACGGCCGCG	TOGTOGROGA	TOCCGGCCAT	GTGCACGACG	GCGCGCAGCC
10	34261	GCTGGGCGAC	GTCGGGCGACG	ACTGCGGCCA	GCTCGTCGCG	STOGACGACG	TCGGCGGCCA
	34321	CGTACCGCAC	GCGGTCGTCC	TOCGGCGTGT	0000033000	SCCGTTGCGG	GACACCACGA
	34381	CGACCTCGGC	GGCCTCGTGC	ACGGTGAGCA	GGTGGTCCAC	GAGGAGGCGG	CCGAGCCCGC
	34441	CGGTGCCGCC	GGTGACGAGG	ACCOTOCCO		GGAGGTTCCG	GTGGCCGCGG
	34501	COMOMOGGGG	CAGACGGGCC	SCACGREETS	TBCCGTCGGC	GACCCGGACG	
15	34561	0300333333	GAGCCCGGCC	GCTATGGDGG		CICGTCCGCT	TOGATCAGOG
	34621	CGACGCGGCC	GGGATGCTCC	STOTOGGGGG		BECGCCGAGC	_
	34681			GCCACGATGA		CGCCCAGCGC	
	34741	GCCAGGTCTG	CACGGTGGTG	AGCAGGTIBC	ggddd ggddd	COGGGTCCGG	
3.0	34801		GTCGCCGGGT		GGACCACGAC		
20	34861	GCACGTCGGC		CAGTCGGGGA	CGGGTGARGC	GGGCACGGGC	
	34921	TOTOGRACAG	DGCCTCGGCA	TOGGGGTOGG	CGGCCCGCAC	GGTCAGGCTG	
	34981			TCCGTGGCGA		CATGTCGGGG	
	35041	CCAGCAGCAC	GOGCAGOGCG			CCTCACGCCG	
25			GOGGCGGCTCC		AGACCGTCCC		
23	35161		GGGGTGCAGC	CCCGTACCGGG		CTGTTCGGCG GGCCCGGAAC	
			GCGGCCCTCC		TCAGGTCGGC		TCGGCGGGCG
	35281 35341	ACGAGAGCGG GCCAGTCCAC			TGTCCACGCT	CAGCGCTCCG	
		GCGCCCAGGG					
30		CGGTTCCGAC				GTCGACCACC	
50	35521		CTCCGCGATC				
		CCACGAGCGC				CTCGTGGTCG	
			TACCGAGACA			TCGCCGTCGG	
						TTCCGCGTCG	ATCCAGTACC
35		GGTCACGGCG				ACGCGTCGCG	AACGACUAGG
	35821					AGTGAAGCGG	
	35881	CCTCGCCTCG	COGCAGTGTG	CCGGTGACGA	CCGTATGCGC	ATGCCCGGCG	AGCGTGTCCT
	35941	CCAGTGCGGT	GGTGAGCACG	GGATGCGCGC	TGACCTCGAC	GAACGCGCGG	TATCCGCGGT
	36001	CCGCCAGGTG		GCGGCGAACC			TCGTACCAGT
40	36061	AGGCGGCGTC				GGTGGAGAAG	
	36121					CGCGCCGCGG	
		CATGCGCGGT					
		GCAGCTCCTC					
4.5		CGGCGACCTC					
45	36361	CCATGCCGCC	CTGCCCGGCC	AGTTCGGTGG	CGACGAGTOG	GUTGUGCACU	GCGACGACCT
		TOGOGGCGTC					
		AGTGGCCGAC					
		CCATCACCGC					
50		GCCGCTGGGC ACTCGCGGAG					
30	36701	CCCACTGGGA	CCGCTGGGCG	AACACGGGCI	ACT CCACACA	TATETCAGEA	ACGTCGGCGG
		TTCCCGTCAC					
	36943	GCACGACCGC	CCCCTCCCC	ACTICGGCAC	CAUGGGGGAA	GAGCGAGTGG	CCGACCGCGG
	36901	CCGCGGCGCC	7.0707.0000	CCCLCCTCTC	00000000000	CCGCAGTCCC	TCCGGGGTCC
55		GGGCCGACAT					
33		GTGCGGGCGC					
		CGAACGACGA					
	37141	GCAGCAGCCG	GATGTCGCCG	TCCCAGTCGA	CGTGCCGGGA	CGGCTCGTCG	ACGTGCAGCG
	37201	TGCGCGGCAG	GACGCCGTGC	CGCATCGCCA	TGACCATCTT	GATGACGCCG	GCGACGCCGG
60		CCGCGGCCTG					
	37321	GTTCGCGCCC	GTAGGCCACT	TGCAGGGCCT	GGGCCTCGAC	GGGGTCGCCG	AGACGGGTGC
	37381	CGGTGCCGTG	TGCCTCCACG	GCGTCGACGT	CACCCGGCGC	CAGGCCGGCG	TCGGCGAGCG
	37441	CACGCTGGAT	GACGCGCTGC	TGCGCAGGCC	CGTTCGGGGC	GGACAGCCCG	TTCGACGCGC
	37501	CGTCGGAGTT	GACCGCGGAG	CCGCGCACCA	GCGCCAGCAC	GGGGTGGCCG	TGGCGGGTGG

	25.						
	37561	CGTCGGAGAG	CCGCTCCAGC	ACCAGGACAC	oggagaaatt	GGCGAAGCTC	
		CGGTGTCCGC	SAAGGCCTTG	GCACGGCCGT	096333364	CCCGCGCTGC	
		CGACGAAGCC	GGTCGTCGTC	GCCATCACCS		GACCAGGGGG	
5		caccogagog					
3		TGTCGACGGT					CCGGAGAGAA
	3.861	CECTGGTCGG	CGTGCCGGTT	GGCCCGAAAC	GGCCCAGGTC	SACSCCCGCG	
	3/921	GUGTGAACGC	GUCCATGAAT	ACCCCGCTGT	COCTOCCOC	PACGCTTTCG	GGCAGGATGC
		COGCTCGTTC					
10	38041	CCAGCGCCTC	ACGCGGGCTG	ATCCCGAAGA	ACGCGGCGTC	UAAGTCGGCG	GCGCCGGTGA
10		GGAAGCCGCC					TOGTAGAGOG
		CGGCGAGGTC					CCGGAGTCGA
		CCAGCCGCCA					
	38281		CGGCTCGTTC				GCCGGAGCGG
15	30341	CAGGGGCCGG	CTCACCCCGC	CUTTUUTCAT	CUAGUUGG	HUCGAGCGCG	GCCGGTGTCG
15	26401	GGTGGTCGAA	GAUGGCCGTC	GOGGNANGEU	STACCOLLO	TSTCTCGGCG	AGGCTGTTGC
		GCAACCGGAC					
	30521	TOTOGGAGGO	rindgddinna	QCGAGCACGG	0330037350	JGCACACACG	
		GGTCACGATG	GCGGTCGUUG	TOGCGGTCGC	GGTTGTCTT		GCGATGCGGC
20	38641		CTGCCGGACG				GGCACGTCCG
20		CGGCGAGGCT					
		CGCGCACCCG					
		ACATGCCCCA					
	35881	CGTCGAGGAR	GGCGTTGGCG	GCGGCGTAGT	1700110100	55355CT3CCG	ASSACGGCGG
25		CGGCGCTGGA					
43		GCCAGGCGGC					
		CGAGGATGCC					
		TGTGGGCGAG					
		CGGGGGGTGGT					
30		GGCGGGCGAG					
30		GGTTGAGGGG GGAGGGTGTG					
		GGAGGGGAGT					
		GGGCGGTGCG					
		TGAGGGTGTG					
35		GGGTGTGGGC					
20		CGTGTCCCTC					
		GGAGCGGGTT				CAGCACCAGC	
		ACACGACAGG				GACGCCGGCC	
		TGAGGGCGAC					
40		ACGGCAGCTC					
		GTGCCGGATG					
		CGGCATACAC					
		ACTCATAACC					
		TGGCCGGCGG					
45		GGGTCAGGGT					
		CGGTCACCGG					
		CTGCGGTCAC					
		CGGTCTCGTC					
		TGCCCCGCAC					
50	40501	GAACAACACC	ACCACCGTCG	TOGGOGGGCA	GTGCTGTGAC	GGCGGCCAGC	ATCGGATGCG
		CCGCCCCGGT					
		GCCTGTGCTC					
		CCGTGCCCCA					
		GCTCCCAGCC					
55		GCAGCAGCAC					
		CCGCATCCAG					
		CGGTCACCCA					
		TTCCCTTCAG					
		CGTAGTCGAC					
60		CCTCCACCGC					
		TCCACACACC					
		CCCGGCCGGC					
	41281	CCTCCAGGCT	GAGGGCTCCG	GCCACACACG	CCGCCGCGAT	CTCCCCCTGC	GAGTGTCCGA
		CCACAGCGTC					

	13.403			T 0 2 m 2 C T 0 0 0	000000000000000000000000000000000000000	1.0001.000	
						ATCCGACCGC	
	41461					ACACTCCTCC	
	41521	OGARORCOGO	GGAACGGTCC			CACCCACTGG	
ε	41581		GAACACCGTA	CGCGGGCTGAT		ACCCATCACC	
5	41641	COAGCAGCAC	CGCACGGTGA	Chumbhumu	CACGCTCACG		TGCGCGACCG
	41701		CACCCCACCC	JUMIJAUMUMI.	ACCCCCAG	CCGCTCCACC	
	41761		ACCACGAGCC			CCCATCACCA	CCCGACTCCA
	41821	CACGCGACGG		COCTCCAGGA		GTTCGTACCG	
1.0	41881	ACGACGACAC	ACCCGCATGC	GGTGCCCGAT	COGACTORG	CCACGGCCTC	GCCTCGGTGA
10	41641	GCAGCTCCAC	CGCACCGGCC	CACCAGTOCA	CATGCGACGA	CGGCTCGTCC	
	42001	TOTTOGGOGG	GATCCCATGC	OGOATOGCCA	TGACCATCTT	GATGACACCG	
	42061	CAGCCGCCTG	CGCATGACCG	ATGTTCGACT	TGACCGAACC	GAGGTAGAGC	
	42121	GGTCCTGCCC	GTAGGCCGCG		GCGCCTCGAT	CGGGTCGCCC	
, -	42181		CGCCTCCACC	ACGTOCACAT			TTGACCAACG
15	32041	COTGCCGGAT	CACGCGCTGC	TBBBBBBACGE	CGTTGGGGGG		TTGGAGGCAC
	42301	CSTCCTGGTT	CACCGCCGAS	CCGCGGACGA		GGTGTGCCCG	TTGCGCTCGG
	42361	COTCOGAGAGAG	CCGCTCCAGC	ACGAGAACCC		GGCGAAGCCG	
	4211	.v:ggc	GAACGCCTTG	CACCGTCCG"		TICGCGUTGC	CGGGAG. IT
	42481	CCACGAGUTC	TGCGGTGTTC	GCCATGACGG	TGACACCGCC	GACCAGCGCC	
20	42541	CCCCGGCCCG	CAGTGCCTGT	GCCGCCTGGT	GCAGGGGGAC	CAGCGACGAD	
	42601	TGTCGACCGT	GACCGCCGGG	CCCTGAAGTC	CGTACACGTA	CGAGAGGCGC	
	42661	CGCTCGTCTG	CGTCGCCGTG	ACACCGAGCC		CCGGCCGACG	CCGTAGCCCT
	42721	GGTTGAACGC	GCCCATGAAC	ACGCCGGTGT	CGCTCTCCCS	GAGCCTGTCC	
	42781	CGGCGTTCTC	GAACGCCTCC	CAGGAGGTCT		GCGCTGCTGG	
25	42841					GAACCCGGCG	CCGGCCAGGA
	42901		GCGTGTCGTG			STCCGGGTCG	TACAGCGCGT
	42961	CGACGTCCCA	GCCCCGGTCG	GTGGGGAACT	CGGTGATCGC	CTCGGTACCG	GCGGCGACGA
	43021	GCCGCCACAG		GAGGCGACCC		TOGGCACGOO	ATGCCGACGA
	43081	TCGCGACGGG	GTCGCCGGAG	CCGAGGGTCT	GGGCGGTCGC	GGGTGCCGCT	GTCGCGGAGC
30	43141	CGGCGAGGTG				GAACGCGGTT	GACGCGGGCA
	43201	CCCGCAGACC	CGTCCGCGCG				AGCGAGTCGA
	43261	GGCCGTTCTC	GCGGAACGTG	CGGTCCGGGG	AGCAGTGTCC	GGCGCCCGGC	AGGCCCAGGA
	43321	CGGTGGCGAC	GCTGTCGCGG				GCACGGGCCG
	43381	CGGCGAGGCG	GTTCGCCCAC		TGGCGTCGGG		CCGGTCAGTG
35	43441	CGGTGAGGAT			TOGTOGOGGO		GCGGAACCGG
	43501	TCCGGGCCAC	GATGTACGAG	CCGCCGCCCG	CGATGGCCTT	CTCGATCAGG	TCGCCGGTCA
	43561	GCGCCGGCCG	TTCGATGCCG	GGCAGCGCGC	GGACGGTGAC	GGTGGGGAGT	CCCTCCGCGG
	43621	CCCGTGGCCG	GGTGTGGGCG	TCGGCGCCGG	CCGGGCCGTC	GAGCAGGACG	TGCACGAGCG
		CGCCGGGGTT			TGGTCACGTG		GTCTCGTCGC
40	43741	GGAGCAGGCC	GGCGACGGTG	TEGGEGTEET	CCCCGGTGAC	CAGGACCGGC	GCGTCCGGGC
						CCGGGCGTGG	
						CGGCAGCGGG	
						CAGGCGCGCG	
						GGTCTTGCCC	
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						GGCGCTGCGG	
						TTTGGCGGGA	
						CGCCATGCCG	
						GCCCGCGTCG	
50						GGGGAACGAC	
						CCGGAACGCG	
	44461	GACCGAACAC	GCGGTCGCCG	GGGGCCAGGT	CGTCGACGCC	GGGTCCGACT	TCGGTCACGA
						GGTGCCGAGC	
						GACCTCGCCG	
55						AGCGTTCCGG	
	44701	GCGCAGCGCC	CACTGGCGCG	GTCGGCAGGG	GGGTGGTGTC	CGCGCGTACC	AGCCGGGGCA
	44761	CGTAGGCCAC	GCCGGCCCGC	AGCGCGATCT	GGGGTTCGCC	GAGCGAGGCC	GCGGCGGGGA
	44821	CGACGTCGTC	ATCGCCGTCC	GTGTCCACCA	GCACGAACGA	TCCGGGTTCG	GCGGCCTGGC
	44881	GGCGCAGCGC	CTCGTCCCAG	AGCCGGGCCT	GGTCCGCGTC	CGGGATCTCG	GCCGGGCCGA
60	44941	CGCCCACCGC	GCGGCGGGTG	ACGACCGTCC	GGCGGGGTGA	CGGGGTGCCG	GGCAGGTCGC
						GCCGGTGGCG	
						CGCGGTGCGG	
	45121	TGACGTGCCA	GATCTCGTCG	GGCACCTTGA	AGTAGGCGAG	CCGGCGGCGG	CACTCGGCGA
	45181	GGATCGCCTC	GGCGGGGACG	CGGGGGCCGT	CGGAAACGAC	GTAGAGCACG	GGTATGTCGC
	•						

					CGGCGTCCCG	GACACCGGCC	ACCTCCTGGG
		CGACGGTCTC		GGGTGGATGT	Torrecteded	SCGGATGATC	
	45301	pdpggccggT		TGTCCGGTCT	CGGCCTGACG	TGCGAGGTCC	CCGGTGCGGT
£		ASSAGSESTE	CACGAGCACC	raggegateg		GGCGTGGTAG	
5	45431	GGCTCGGCCC		AGCTCGCCCT		TGCCACGTCG	
	45541	CACCTOSCT	GAACCGCAGC	GACAGGCCCC	GGAGGGGGAG		
	45611	GUGGATGOTO	CASSGTSTTS	GCGGTGAGCG	ASCOSSTOST		
	45661	CGAGMAGGGG	CAGGCCGAAC	GTGGGGTGGA	AATCCCTGGT		GGCGAGGTGG
10	45721	ATCOGGOGAC	CAGCGCCACG	CGCAGCGCGC	GAGCCCGCGG		
10	45791	GGAGGTAGCG	GTACATCGTC		CGAGCACGGT		TOGGOCAGGG
	45841	CSTCGAGGAG	GTCACGCGCG		CCAGGATACS		CCGACCGTGA
		GGACGGCGAG	CAGGCAGAGG		GGCTGTGGAA		GGCCAGAGCA
		GTTCGTCGTC	CTCGGTCAGC		GCACGTCGCA		
15	46021	CGCTGCGCTG		ACGCCCTTGG			GTGTAGAGCA
1.7	46081	TOCAGGGGG		CCGAGGTCGT	00000000000000000000000000000000000000	GCACGGCGGC	
	46141						
	46201	COSTGCCSGT	GCGGCGCACC	TGGTCGAGGT	GGGTTTCGTC	GGTGACCAGC	
	4/261	CGGAGTCCCT	n-9GAAL.su	330 00000	AGACCTOGAT		
20	46321	CGACGGCGGC GCAGCATCGC	GGCGCGGGCG GACCCGGTCG		CGCCGGACGC	GGTCTCGATC GGCGAGGTGT	CGGTTGCCGA
20			GAGCCGGAGT		TCACGGCGCG	TOGGGAATCC	CCGGCGAGCC
	46501		GCGTCGCTCG		GGAGCAATTC		
	46561		CATGGAAACA			STGCAACGGC ACAACAGCAC	
			GCCGGCGACG			GOCACCCCCT	
25	46681	CTACCGTGGC				GCACGCATAC	
					CCAGGGAAGG		
	46801		CCGTATTGCC			COGGACCTCG	
		GACGGTGCTC				SACGTGTCGC	
		TGCTCCCCGG				GAGGCGTTCA	
30					GGACAACGGC		
					CGAGAGCGGC		
					CCGGTCGCTG		
	47161	GACCTGGAAC	TACGTCAGCG	GTATCAACAC	GACGAACGCG	GACGGGCTGG	AGGTGTACCG
	47221	GGACTTCTGC	GTGGGCCGCG	CCCAGGCGCT	CGACGAGGGC	GGGATCGACC	CGGCCACCAT
35	47281	GCCCGCGGCC	ACCGGTATCG	GCGCCCACGG	GGGCGGCATC	ACCTGCGTGT	TCCTCGCCGC
	47341	CCGGGGGGGA	GTGCGGATCA	ACATCGAGAA		CTCACGGCCC	
	47401	GACGACGTAC	GGTCCGCGGC	CCCCGGTCTT	CGCACGGGGC	ACCTGGCTGG	GCCCGCCGCA
	47461	GGGGGGCCGG	CTGTTCATCT			GGACACCGAA	CGGTGCACCA
		CGGTGATGTG			CCTCGACAAC		TCATCGGCGC
40					GGGGCACGTC		TGGACCACCT
					CGATACGGTC		
					GCACACCGAC		
					ATACCCGGTA		
4.5					GTCACCGCAC		
45					CTCCAGCAAT		
					GGTCTATTGG		
					GCGTTGCGGA CCGGAACACC		
					CGCGACGAGA		
50					ATCCGCCTCG		
30	40101	CCCACCOGGC	TOTTONICAG	CCCCCCCCC	GGCTGGTCGT	TOSCOCOTOCT	CCAACATCAA
					ACTGCCCGCC		
					GAGCGGCGCG		
					CTCCGGGGGG		
55					GACGCGGACG		
55					ACGCTCGCGC		
					GTCCTCGCCC		
					ACGCGGGCGG		
	48721	ATCTTCCTCA	ACACCCTCCC	CCTCCCCCCC	GACCTCTCGG	GCGATCCGTC	GTTCCGGGAA
60					GACGCGTTCG		
	48841	GAGAACGTCA	TCGAACTCGT	CGCACCGGAA	CGCGACCTGT	CGGTCAACCC	GGTCGTCCAG
	48901	GTGCTGTTGC	AGGTGCTGCA	GCGCGACGCG	GCGACGGCCG	CGCTGCCCGG	CATCGCGGCC
	48961	GAACCGTTCC	GCACCGGACG	CTGGTTCACC	CGCTTCGACC	TCGAATTCCA	TGTGTACGAG
	49021	GAGCCGGGTG	GCGCGCTGAC	CGGCGAACTG	CTCTACAGCC	GTGCGCTGTT	CGACGAGCCA
	*					7	

	10001	CGGATCACGG	Commonmoon	COrcencaco	COCCEOTEC	: cccccmanc	acacanacac
	49951	GACGTACGGC	MCMCCCCC	GONGLICADG	01000100110	AGGCGG1CAC	CCCCCCCCCCCC
	49201				GACACGCTGC	CGGGCCTGCT	GGCCCGGTAC
		TOGAACGACA GOOGGACGCA	CCCCCGGGGG			ACATCTCCCT	CACCTACGCG
5	49301	GAGCTGGACG	GGCGGGGGAA		CACCIGOTOC		CACCGCCACC
٠,	49351	GGCGACCTGG		CGGGGATGGC	GGCGCCGACC		CATCGTGGGG
		ATCOTOANGG		TTATGTGGGG		AACATOOTOO	GGAGCGCACG
	49501		TGGCCGACGC		ACGGTGGT30	CGCACGAGGT	CTACCGTTCC
		cogrectors		COTOSTGGCG	TTGGACGACC		CCGGCAGCCG
10	49631	GACGACACGG		CGAGCTGGAC	CGGGACAGCC	TOGCCTACGC	GATCTACACG
- 0	49681	TOCGGGTCGA		GAAGGCCGTG	CTCATGCCGG		CGTCAACCTG
		CTGCTCTGGC		GATGGGCCGC	GAGCCGGCCA		CCAGTTCGTG
	49801		TCGACTACTC		ATCTTTTCCG		CGGCACGCTC
		GTCATCCGGC			CCGCCGGGAC		GATGGACGAA
1.5		CAGGCGATTA			GCCGTACTGC		CGAGCACGTC
		GATOCGCACA		CSCCSCCCTG		GCCAGGGCGG	CGAGGCGCTG
		ATCCTCGACG		CGAGCIGTGC		CCCACCTGCG	CGTGCACAAT
		CACTACGGTC			ACCGGGTACA	CGCTGCCCGC	CONCCCGAC
	50161	GCGTGGCCCG	CCACCGCACC	GATEGGECCG	COGATOGACA	ACACCCGCAT	CCATCTGCTC
20		GACGAGGCGA			ATGCCGGGGG	AGCTCTGCGT	CGCCGGCGTC
	50281	SGCCTCGCCC	GTGGGTACCT	GGCCCGTCCC	GAGCTGACCG	CCGAGCGCTG	GGTGCCGGGA
	50341	GATGCGGTCG	GCGAGGAGCG	CATGTACCTC	ACCGGCGACC	TGGCCCGCCG	CGCGCCCGAC
	50401	GGCGACCTGG	AATTCCTCGG	CCGGATCGAC	GACCAGGTCA	AGATCCGCGG	CATCCGCGTC
	50461	GAACCGGGTG	AGATCGAGAG	COTGOTOGOO	GAGGACGCCC	GCGTCACGCA	GGCGGCGGTG
25		TCCGTGCGCG				CGTACGTCGT	
	50591	GGCCGGCACG	GCGACGACTT	CGCCGCGTCG	CTGCGCGCGG	GACTGGCCGC	COGGCTGCCC
	59641	GCCGCGCTCG	TGCCCTCCGC	CGTCGTCCTG	GTGGAGCGAC	TGCCGAGGAC	CACGAGCGGC
		AAGGTGGACC			GAGCCGGGGCC	CGGCGTCGAC	CGGGGCGGTT
		ACGCCCCGCA				TCCAGGAGGT	
30		CCGCGGGTCG				GGCACTCCCT	GCTCGCCACC
		CGGGTCGTCT					TACGCTCTTC
	50941	GACGGGCGGA					
	51001		CGCCCTCCGC				
2.5		ATGCTGCACT					
35		TTCCGGCTGC					
		GOGOGOCACG					
	51241		GCGCCGAGGT				
		GTCGCCCACC					
: 40		GTGCTGCTGC					
40	51421	GGTGACGGAT	GGTCCTTCGA	CCTCCTGGTC	CGGGGAGITGI	CGGGGACGUA	ACCEGACUTT
	51481	CCGGTGTCCT	ACACGGACGT	GGCCCGGTGG	GRACOGRATI	CGGCCGTGAI	CGCGGCCAGG
	51541	GAGAACGACC GCGGTCCGGC	GGGCCTACTG		CIGGGGGGGG	TOMOCACCOT	CENCCECNCC
		GCCGTCCTGG					
45		CTCGGCGCCT					
40		ACGCCGTTCG					
		GTCCTCGCGC					
	51901	GT:GCACACCG	CGATGGTGGG	CGCGCACGCC	CACCAGGCGG	TECCCTACTC	CGCGCTGCGC
	51961	GCCGAGGACC	CCGCGCTGCC	GCCGCACCCC	GTGTCGTTCC	AGCTCATCAG	CGCGCTCAGC
50		GCGGAACTGC					
20		GACGAGATGA					
		GCGGTGGTCC					
		GTGGAGGCGA					
		GAAAGCGAGT					
55		CGGAACTCCA					
		GGATGGCCTG					
		AGTCCGGTGG					
	52501	ACGGTCGCGG	CGGCTTCCTC	ACCGGGGCGG	CCGGCTTCGA	CGCGGCGTTC	TTCGGCATCA
	52561	GCCCGCGCGA	GGCGCTGGCG	ATGGACCCGC	AGCAGCGCCT	GGCCCTGGAG	ACCTCGTGGG
60	52621	AGGCGTTCGA	GCACGCGGGC	ATCGATCCGC	AGACGCTGCG	GGGCAGTGAC	ACGGGGGTGT
		TOCTOGGGGGC					
	52741	CGAGCATTCA	CACGAGCGTG	CTCTCCGGCC	GCCTCGCGTA	CTTCTACGGT	CTGGAGGGTC
	52801	CGGCGGTCAC	GGTCGACACG	GCGTGTTCGT	CGTCGCTGGT	GGCGCTGCAC	CAGGCCGGGC
	52861	AGTCGCTGCG	CTCCGGCGAA	TGCTCGCTCG	CCCTGGTCGG	CGGCGTCACG	GTGATGGCCT

	52921	0500660666	GTTCGCGGAS	TTOTOGGAGO	AGGGGGGGCT	GGCCCCGAC	GCGCGCTGCA
	52981	AGGCCTTCGC	GGAAGCGGCT	GACGCCACCS	STITCGCCGA	agggreege	GTCCTGATCG
	53040	TOGAGAAGOT	CTCCGACGCC	GAGCGCAACG	GCCACCGCGT	SCTGGCGGTC	GTCCGGGGTT
	53101						
ε		COBCOGTCAA	CCAGGACGGT	GCCTCCAACG	GGCTGTCCGC		CCGTCGCAGG
5	5316:	AGCGGGTGAT	CCGGCAGGCC	CTGGCCAACG	COGGACTICAC	CCCGGCGGAC	STGGACGCCG
	53211	TUBAGGGGGA	0390700330	ACCAGGCTGC	acamagair.	COAGGCACAG	GCCGTGCTGG
	53251	20ACCTACGG	GCAGGGGGGC	GACADGCCTG	TGCTGCTGGG	STEGETGAAG	TOCAACATOG
	53341	GCCRCRCCCA	9900900000	9906703003	GTGTCATCAA	GATGGTCCTC	GCCATGCGGC
	53401	HOSGOAGCOT	scoccecace	OTSCHOOLS	ACACGCCCCC	CTCGCACGTC	GACTGGACGG
10	£3461	cosciscos	CGNACTOCTO		GOCCTGGGG	CGAAACCGAC	CGCCCACGGC
• •	53521	GCGCCGGTGT	CTCCTCCTTC	GSCGTCAGCG	GCACCAMCGC	CCACATCATC	CTCGAAAGCC
	53581	ACCCCCGACC	GGCCCCGAA	0000000033	CACCCGACAC	CGGACCGCTG	CCGCTGCTGC
	53641	TOTOGGGGGGG	CACCCCGCAG	GCACTCGACG	CACAGGTACA	CCGCCTGCGC	GCGTTCCTCG
	53701	ACGACAACCC	CGGCGCGGAC	CGGGTCGCCG	TOGOGCAGAC		CGCACCCAGT
15	53761	TOGAGDACOG	CGCCGTGCTG	CTCGGCGACA	CGCTCATCAC	CGTGAGCCCG	AACGCCGGCC
	53531	SCGGACCGGT	GGTCTTCGTC	TACTCGGGGG	AAAGCACCCC	GCACCCGCAC	A.CCGGGCGGC
	53881	AACTCGCGCC	CACCTACCCC	GTGTTCGTCG	AAGOGTBBCD	CGAGGCCCTC	SACCACCTCG
	53941	- 22221010	36600003337	ACCOACTICG	0001021	CGCGCTCACC	GOGCTOCTGC
	00041	AUGULNOUGA			DOUBLILMSAU		
20	1400.	36.46.10000	CATCACCCCG	CACGCGGTCA	TOUGUEARTE		ATCACCGCCG
20	54061	DGCACGCCGC	CGGTGTCCTG	TOCCTGAGGG	MCGCGGGGGG		ACCOGCACUC
	54121	GOCTGATGGA	CCAACTGCCG	TCGGGCGGCG	CGATGGTCAC		AGCGAGGAAA
	54181	AGGCACGCCA	GGTGCTGCGG	CCGGGGGGTGG	AGATOGCCCC	COTCAACGGC	CCCCACTCCC
	54241	TIGIGOTOTO	CGGGGACGAG	GAAGCCGTAC	TCGAAGGCGC	COGGCAGOTO	GGCATCCACC
	54301	ACCCCCTGCC	GACCCGCCAC	GCCGGCCACT	CCGAGGGGAT	GCAGCCACTC	GTCGCCCCCC
25			GGCCGGGACC		ACCAGOGGA		CCCGGCGACC
20	54361	TOOTOGAOGT		CTGACCTACC		CACCGCCATC	
	54421	CCACCACCGG	CGANTACTGG	GCGCACCAGG	TOOGGGACCA	AGTACGTTTC	CAGGCGCACA
	54481	CCGAGCAGTA	cccgggeges	ACGTTCCTCG	AGATOGGOOD	CAACCAGGAC	CTCTCGCCGC
	54541	TOGTOGACGG	CGTTGCCGCC	CAGACCGGTA	CGCCCGACGA	GGTGCGGGCG	CTGCACACCG
	54601	CGCTCGCGCA	GCTCCACGTC	CGCGGCGTCG	CGATCGACTG	GACGCTCGTC	CTCGGCGGGG
30	54661	ACCGCGCGCC	CGTCACGCTG	CCCACGTATC	CGTTCCAGCA	CAAGGACTAC	TGGCTGCGGC
	54721	CCACCTCCCG		ACCGGCGCGG	GGCAGGAGCA	GGTGGCGCAC	CCGCTGCTCG
	54781	GCGCCGCGGT	CGCGCTGCCC		GAGTCGTCCT		CTGTCGCTGG
							=
	54841	CCTCCCATCC	GTGGCTCGGC	GAGCACGCGG	TCGACGCCAC	CGTGCTCCTG	
2.5	54901	CCTTCCTCGA		CGCGCCGGCG		CTGCGACCTG	
35	54961	TEGTEATEGA	GACGCCGCTC	GTGCTGCCCG	CGACCGGCGG	TGTGGCGGTC	TCCGTCGAGA
	55021	TOGCOGAACO	CGACGACACG	GGGGGGGGG	CGGTCACCGT	CCACGCGCGG	GCCGACGGCT
	55081	CGGGCCTGTG	GACCCGACAC	GCCGGCGGAT	TOCTOGGCAG	GGCACCGGCA	CCGGCCACGG
	55141	CCACGGACCC	GGCACCCTGG	cogodogogo	AAGCCGGACC	SSTCGACGTC	GCCGACGTCT
	55201	ACGACCGGTT	CGAGGACATC	GGGTACTCCT	ACGGACCGGG	CTTCCGGGGG	CTGCGGGCUG
40	55261	COTGGCGCGC			AGGTCGCGCT		CAGAGCGCCG
40			CGGCGACACC				
	55321	ACGCCGCCCG			TGCTCGACGC	CGCGTTCCAG	
		TGGCCGCGCT					
		GCATCCACGC					
	55501	GCACCGTCCG	CATGACCGGC	CCGGACGGGC	AGCTGGTGGC	CGTGGTCGGT	GCCGTGCTGT
45	55561	CGCGCCCGTA	CGCGGAAGGC	TOOGGTGACG	GCCTGCTGCG	CCCGGTCTGG	ACCGAGCTGC
		CGATGCCCGT					
		ACGGCGACGT					
	55741	GCCACCTGTC	0000000000	CACACCACCA	TONECOTOCO	6366666766	GGCCCGGCCG
70	22801	CTGCCGCCGC	CGCGGGTCTG	GTCCGCTCGG	CGCAGGCGGR		0606106160
50		TCGTCGAGGC					
		AACCGCAGCT					
	55981	ACCCCGCGCA	CGGCCCGCTG	TCCCTGCCGG	ACGGCGACTG	GCTGCTCACC	CGGTCCGCCT
	56041	CCGGCACGTT	GCACGACGTC	GCGCTCATAG	CCGACGACAC	GCCCCGGCGG	GCGCTCGAAG
	56101	CCGGCGAGGT	CCGCATCGLC	GTCCGCGCGG	CCGGACTGAA	CTTCCGCGAT	GTGCTGATCG
55		CGCTCGGGAC					
))							
		AGACCGGGCC					
	56281	GCGGCATCGG	CCCGACGGCC	GTCACCGACC	GGCGCTGGCT	GGCCCGGATC	CCCGACGGCT
	56341	GGAGCTTCAC	CACGGCGGCG	TCCGTCCCGA	TCGTGTTCGC	GACCGCGTGG	TACGGCCTGG
	56401	TCGACCTCGG	CACACTGCGC	GCCGGCGAGA	AGGTCCTCGT	CCACGCGGCC	ACCGGCGGTG
60		TCGGCATGGC					
		GTACCGGCAA					
	56501	CTCGGACGAC	CCCCGGCCCC	ACCCCTTTCC	CGCGCATGGA	CGTCGTCCTG	AACGCGCTGA
	50001	CCCCCCACCAC	CGCG11CCGG	MCCGCITICC	TCCTCCTCCC	0010010010	TTCCTCCACA
	565041	CCGGCGAGTT	CATCGACGCG	TUGUTUGAGU	TOUTGOMCOC	COCCER CCEC	CCCmmccacc
	56/01	TGGGCCGCAC	CGAGCTGCGC	GACCCGGCCG	CGATCGTCCC	CGCCTACCTG	CCGTTCGACC

				2222			
	56761	TGCTGGACGC			AGATCCTGGG	CGAACTGCTC	
		ACGCGGGCGC		crecesered	GTGCCTGGGA		GCACGCGACG
	56881	CGCTCGGCTG	GATGAGCCGC	GCCCGCCACA	TOGGCAAGAA		CTGCCCCGGC
_	56941	CGCTCGACCC	GGAGGGGGCC	STOGTOCTCA	CCGGCGGCTC		GCCGGCATCC
5	57001	TOGOCOGOCA	CCTGCGCGAA	COGCATGTCT	ACCTGCTGTC	COGGACGGCA	
	57061		CGTCCACCTG	CCCTGCGACG	TOGGTGACCG		GCGGCGGCCC
	57121	TGGAGCGGGT	GRACOGGCCG	ATGACCGCCG	TGGTGCACCT		CTGGACGACG
	571,51	GCACCGTCGC	GTOGOTOAGO	CCCGAGCGTT	TOGACACGGT		AAGGCCGACG
10	57241		COTOCACGAG	CTGACGAAGG	AGCAGGACCT	CGCCGCGTTC	GTGCTCTACT
10	57301	CGTCGGCCGC	CGGCGTGCTC	GGCAACGCCG	GCCAGGGGAA	CTACGTCGCC	GCGAACGCGT
	57361	TOOTCGACGO	GCTCGCCGAG	CTGCGCCACG	GTTCCGGGGT	SCOGGCCCTC	TCCATCGCCT
	57421	GGGGGCTCTG	GGAGGACGTG	AGCGGGGTCA	COGCGGGGGGT	CGGCGAAGCC	GACCGGGACC
	57481	GGATGCGGCG	CAGCGGTTTC	CGGGCCATCA	CCGCGCAACA TCGCGGCGGGC	GGGCATGCAC	CTGTACGAGG
15	57541	CGGCCGGCCG	CACCGGAAGT	CCCGTGGTGG	CCGTCCGGCG		GCGCCGGACG
15	57601	TGCCGCTGCT	GCGCGGCCTG	GGGGGGAGGA GGGGTGAGGG	GCGACGAGGT	CGCCGAAGCG	CGGGAGTGTT
	57661	CGTCCGCCGA	CCGGCTCGCC	GCCGTGCTCG	GCCACGTGGG	TGGCGAGGAC	
	57721 57781	TOGTOCGGGA CGGCGGCGIT	GAGCACCGCC	GGCATCGACT	CGCTGACTGC		CGCAACGCCC
			CAAGGA ICTC GACCGGTGTG	CGGCTGAACG	CCACGGCGGT		CCGACCCCGC
20	5784 57901	CACCGAGGC ACGTGCTCGC	CGGGAAGCTC	BBCGACGAAC	TGACCGGCAC	CCGCGCGCCC	
0 ش	57961	GGACCGCGGC	CACGGCCGGT	GCGCACGACG	AGCCGCTGGC	GATCGTGGGA	
	58021	GGCTGCCCGG	CGGGGTCGCG	TCACCCGAGG	AGCTGTGGGA	CCTCGTGGCA	TCCGGCACCG
	58081	ACGCCATCAC		ACGGACCGCG	GCTGGGACGT	CGACGCGATC	TACGACCCGG
	58141	ACCCCGACGC	GATCGGCAAG	ACCTTOGTCC	GGCACGGTGG		GGCGCGACAG
25	58201	GCTTCGACGC	GGCGTTCTTC	GGCATCAGCC	CGCGCGAGGC	COTOGOGATG	GACCCGCAGC
	58261	AGCGGGTGCT	COTGGAGACG	TCGTGGGAGG	CGTTCGAAAG	CGCCGGCATC	
	58321	CGACCCGCGG	CAGCGACACC	GGCGTGTTCG	TCGGCGCCTT		TACGGCACCG
	58381	GTGCGGACAC	CGACGGCTTC	GGCGCGACCG	GCTCGCAGAC		TCCGGCCGGC
	58441	TGTCGTACTT	CTACGGTCTG	GAGGGTCCGG	CGGTCACGGT	CGACACGGCG	TGTTCGTCGT
30	58501	CGCTGGTGGC		GCCGGGCAGT	CGCTGCGCTC	CGGCGAATGC	TOGOTOGOCO
	58561	TGGTCGGCGG	CGTCACGGTG	ATGGCGTCTC	CCGGCGGCTT	CGTGGAGTTC	TCCCGGCAGC
	58621	GCGGCCTCGC	GCCGGACGGC	CGGGCGAAGG	CGTTCGGCGC	GGGTGCGGAC	GGCACGAGCT
	58681	TCGCCGAGGG	TGCCGGTGTG	CTGATCGTCG	AGAGGCTCTC	CGACGCCGAA	CGCAACGGTC
	58741	ACACCGTCCT	GGCGGTCGTC	CGTGGTTCGG	CGGTCAACCA	GGATGGTGCC	TCCAACGGGC
35	58801	TGTCGGCGCC	GAACGGGCCG		GGGTGATCCG	GCAGGCCCTG	
	58861	GGCTCACCCC	GGCGGACGTG	GACGCCGTCG		CACCGGCACC	
	58921	ACCCCATCGA	GGCACAGGCG	GTACTGGCCA	CCTACGGACA		
	58981	TGCTGGGCTC	GCTGAAGTCC		ACGCCCAGGC	CGCGTCCGGC	
. 40	59041	TCATCAAGAT	GGTGCAGGCC	CTCCGGCACG	GGGAGOTGCC		CACGCCGACG
40	59101					ACTGCTGACG	
	59161	CGTGGCCCGA	GACCGACCGG	CCACGGCGTG	CCGCCGTCTC	CTCGTTCGGG	
	59221	CCAACGCCCA	CGTCATCCTG	GAGGCCGGAC	CGGTAAUGGA	BACGCCCCCC	GCATCGCCTT
	59281	CCGGTGACCT	TCCCCTGCTG	GTGTCGGCAC	GCTCACCGGA	AGCGCTCGAC	GAGCAGATCC
15	59341	GCCGACTGCG	CGCCTACCTG	GACACCACCC	CGGACGTCGA	CCGGGTGGCC	DOCOMONMON
45	59401	CGCTGGCCCG	GCGCACACAC	TTOGCCCACC	GCGCCCCCCC	GCICGGIGAC	CACCCACC
	59461	CCACACCCCC	CGCGGACCGG	CCCGACGAAC	COCCOCA MCC	COTOTTO	CAGGGCACCC
	59521	AGCATCCCGC	GATGGGCGAG	CAGCTCGCCG	CCGCCCATCC	CGTGTTCGCC	DECENCENTE
	59581	ATGAAGCGCT TGCTCTTCGC	CCGCCGCCTI	GACAACCCCG	CCCTCCTCCC	CTCCTCCCCC	AGCCAGCAIG
50	59641	ACGCGGTCAT	CCACCAGGCG	GUGITUAUUG	TCC1CC1GCG	CCACCCCCC	CCCATCCTCT
50	59701	CGCTGGACGA	CGGCCACICG	CIGGGGGAGA	CCCCCCCCCC	CCTCATCCAC	A COCTCCCC
	50001	CACCCGGTGC	CARCORCACC	CIGATOACCA	GEDGESCOG GEDGESCOG	GCCACGCCAG	GCGTTGCGGC
	50001	CGGGCGTGGA	CATGGTCACC	CTCT TCGGGG	CCCACCCCAT	CGTGCTGTCC	GGGGACGAGG
	22001	ACGCCGTGCT	GAICGCCGCC	COCCARCORCO	GCATCCACCA	CCGCCTGCCC	GCCCCGCACG
55	50001	CCGGGCACTC	CUCCOTCOCC	CYCCCCCTCC	CCGCCGAGCM	GCTCGCCACC	ACCCGCGGGC
טע	80081 00001	TCCGCTACCA	COCOCACATO	20100000100 2017012770	CGAACGACCC	CACCACCGCT	GAGTACTGGG
	60101	CCGAGCAGGT	-0001000AC	Castacatte	ACGCCCACGC	GCAGCAGTAC	CCGGACGCCG
	60121	TGTTCGTGGA	GATOGGCCCC	GCCCAGGACC	TCTCCCCCCCT	CGTCGACGGG	ATCCCGCTGC
	60241	AGAACGCCAC	CGCGGACGAG	CTGCACGCGC	TGCACACCGC	GCTCGCGCAC	CTCTACGCGC
60	60241	GCGGTGCCAC	CCTCGACTGG	COCCGCATCC	TOGGGGCTGG	GTCACGGCAC	GACGCGGATG
	60361	TGCCCGCGTA	CGCGTTCCA	CGGCGGCACT	ACTGGATCGA	GTCGGCACGC	CCGGCCGCAT
	60421	CCGACGCGGG	CCACCCCGTG	CIGGGCTCCG	GTATCGCCCT	CGCCGGGTCG	CCGGGCCGGG
	60481	TGTTCACGGG	TTCCGTGCCG	ACCGGTGCGG	ACCGCGCGGT	GTTCGTCGCC	GAGCTGGCGC
	60541	TGGCCGCCGC	GGACGCGGTC	GACTGCGCCA	CGGTCGAGCG	GCTCGACATC	GCCTCCGTGC

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						GGTCGACGAG	
			CCGGTTCACC				ACGCTGCACG
	60721	COGAGGGGGT	GCTGCGCCCC		COCTCCCGA	TGCGGCCGAC	
-	60781	CCCCACCGGG	CGCGGTGCCC		TGCCGGGTGT		GGGGACCAGG
5	60841		GGCCGAGGTG	GACGGACCOG	ACCOTTTCGT		GACCTGCTCG
	60901	ACGCGGTCTT	CTCCGCGGTC	GGCGACGGNA	GCCGCCAGCC		CGCGACCTGA
	E0961		GTC3GACGCC	ACCGTACTGC	3030013301	SACCOGGGGG	ACCGACGGAG
	61021	COATGGGATT	CGCCGCCTTC	GACGGCGCCG	accrecesan	ACTCACCGCG	GAGGCGGTGA
	61081	CGCTGCGGGA	GGTGGCGTCA	COGTCCGGCT	COGAGGAGTO	GGACGGCCTG	CACCGGTTGG
10	61141	ASTGGCTCGC	SGTCGCCGAG	GOGGTOTAGG	ACGGTGACCT	GCCCGAGGGA	CATGTCCTGA
	61201	TCACCGCCGC	CCACCCCGAC	GACCCCGAGG	ACATACCOAC	COSCGCCCAC	ACCCGCGCCA
	61261	COCCOGTCCT	GACCGCCCTG	CAACACCAGO		CGACCACACO	CTCATCGTCC
	61321	ACACCACCAC	CGACCCCGCC	GGCGCCACCG		CACCCGCACC	
		AACACCCCCA		CTCATCGAAA	CCGACGACCC		CTCCCCCTGG
15	51441		CACCCTCGAC	CACCCCCACC	TOOGCOTOAC		
	61501		CCCCCTCCAC		CACCCACUAC		AACCCCGAAC
	-	ACGCCATCAT	CATCACCGGC	GGCTCCCG			CGCCACCTGA
		ACCACCCCA	CAUCTACCTC		.000000	TGACGCCACC	CCCGGCT.CCC
	01681	ACCTCCCCTG			AACTCGCCAC		CACATCCCC
20	61741		CGCCATCTTC		COACCCTCGA		CTCCACGCCC
~0	61801		CCGCCTCACC		ACCCCAAAGD		TGGCACCTGC
		ACCACCTCAC			ACTTCGTCCT		GCCGCCJCCG
	61921					DECOTTODE	
	61981	1001000CA0	CCCCGGACAA	GGAAACTACG	CCGCCGCCAA	0000110010	
25		CCACCAGCAC					ATGTGGCACA
20					TGCGCCTCTA		CGCCGCGGGG
	62161						GTCGGCTCCG
	62221		GAGCGGCCTG		ACCCGGCACA CGCGGCGCGT	CGCCCGTGCC	GGCTCCGTAC
	62281				CCGACCGCGG		GGGCAGACGT
30	62341				ACGCCGACGC		ACCACCCTCG
50							GCGCCGACCA
	62461					CGAGCTGCGC	
						CGACCACCCG	
	62521		CAAGCTCCGC				CCCGCGCGGA
35					CGATCGTCGG		CGACTGCCCG
33		GCGGGGTCGC				GTCCGGCACC	
		CCGAGTTCCC				GTTCGACCCG	
	62761	CCCCCGGCAA		CGGCACGGCG			GGCTTCGATG
	62821						CAGCGCGTCA
40	62881	TOOTOGAAAC		GOGTTCGAGA			ACGCTGCGCG
40	52941					STACGGCGCC	
	63001		CGGCGCCACC			CTCCGGCCGG	
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						CTCGCTGGCG	
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45						CGGCACGAGC	
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						CTCCAACGGC	
						CGACAAGGCC	
• •						CCCGCTGGGC	
50						CACACCGCTC	
						TGTCGCCGGC	
						GCACGTGGAC	
						CGAGGCGAGG	
						TATCAGCGGT	
55	63841	ACGTGATCCT	TGAGGGTGTT	CCCGGGCCGT	CGCGTGTGGA	GCCGTCTGTT	GACGGGTTGG
	63901	TGCCGTTGCC	GGTGTCGGCT	CGGAGTGAGG	CGAGTCTGCG	GGGGCAGGTG	GAGCGGCTGG
	63961	AGGGGTATCT	GCGCGGGAGT	GTGGATGTGG	CCGCGGTCGC	GCAGGGGTTG	GTGCGTGAGC
	64021	GTGCTGTCTT	CGGTCACCGT	GCGGTACTGC	TGGGTGATGC	CCGGGTGATG	GGTGTGGCGG
	64081	TGGATCAGCC	GCGTACGGTG	TTCGTCTTTC	CCGGGCAGGG	TSCTCAGTGG	GTGGGCATGG
60	64141	GTGTGGAGTT	GATGGACCGT	TCTGCGGTGT	TOGOGGCTOG	TATGGAGGAG	TGTGCGCGGG
	64201	CGTTGTTGCC	GCACACGGGC	TGGGATGTGC	GGGAGATGTT	GGCGCGGCCG	GATGTGGCGG
	64261	AGCGGGTGGA	GGTGGTCCAG	CCGGCCAGCT	GGGCGGTCGC	GGTCAGCCTG	GCCGCACTGT
	64321	GGCAGGCCCA	CGGGGTCGTA	CCCGACGCGG	TGATCGGACA	CTCCCAGGGC	GAGATCGCGG
	64381	CGGCGTGCGT	GGCCGGGGCC	CTCAGCCTTG	AGGACGCCGC	CCGCGTGGTG	GCCTTGCGCA

	51171	GCCAGGTCAT	concentration.	organeassa	202222007		CCAMMCCCCC
	64511	COGGTGAGGT	CGGGGGGGA	01000003330	SSATCGCCCC	GCGTAACGGC	
		CAGTOSTGGC	CGGCGAGCCG	mrsscssmaa	LOGACOTOCT		GAGACCGAAG
	64621	COGTGCGAGT			ADSCOTOCOA	CACGCCCCAC	GTGGAAGCCA
5	64681		ACTOSCIGAG			GAAGGCCGCG	TOGGTGGCGT
٥	64741	GGTGGTCGAC		GUCTGGGTGA		GGATGAGAGT	TACTGGTACC
		GGAACCTGCG			CGGCGGTCGC		GGGTCCGTGT
	64861			CCGGTGCTGC	TGCCGGCGAT		CACACGGTGG
	54921	CGTCGTTGCG		GGCGGCTGGG		SACGGCGTTG	GCGCAGGCGT
10	64981	GGACCCTGGG		GACTGGGACA			GGGCGGCTGC
	65041	TOGATOTOGO			GGCGCTACTG		GCCGGTGCCA
	65101	CCGACCTGTC	cacagacaga	CTGACAGGGG	CAGCACATCO	CATGCTGGCC	GCCATCACGG
		CACTACCCGC	CGACGACGGT		TCACCGGCCG	GATCTCGTTG	
	65221	COTGGCTGGC	TGATCACGCG	GTGCGGGGCA	CGGTCCTGCT	GCCGGGCACG	GCCTTTGTGG
15	65281	AGCTGGTCAT	COGGGCCGGT	GACGAGACCG	GTTGCGGGAT	AGTGGATGAA	CTGGTCATCG
	65341	TOCCCCCT	CGTGGTGCCG	GCGACCGCAG	CCGTGGATCT	STCGGTGACC	GTGGAAGGAG
	65401	CTGACGAGGC	CGGACGGCGG	CGAGTGACCG		CACCGAAGGC	ACCGGCAGCT
	65461	GGACCCCCLCI.	CEDDAGGOCAGO	AUTOTGACCC	COGACACCCC	CGACACCCCC	AACGCTTCCG
	65521	GTGTTGTCGG	TGCGGAGCCG	TTCTCGCAGT	GGCCACCTGC	CACTGCCGCG	
20	65561	CCTCSGAGTT	CTACTTGCGC	CTGGACGCGC		GTTCGGACCC	
	65641	GAATGCGGGC		GATGGTGACA			CTCCCCGAGG
	65701	ACCGTGCCGC				GCTGCTCGAC	
		AGAGCGGCAG	CCTGCTCATG			GAGOGTGCAA	
2.5	65821				GOGOGACCAT		
25		CGGGCCCGGA					
	65941	TOGACGCGCT		TCCCCGGAAG			CCGATGCTGC
		GGGTCGGGTG		COOGTACCTG			GCGGACGTGC
		TGACGCTGCG				COGGGACCTG	
30		TTCTCGACGC	GCTGCTCCGG				GGCGCCTCG
30		CCGCCAAGGC	GGCCGCAGGC AACGGACCCG				GCGATCGCGG
	66241		GCCCCATGTG				CGGCTGATGC
		CACTOGGCGA GGGCCACGCC				GCAGCTGCGG	
		CCGGTTCCCT				CCCGGACCGG	
35	66481		CGACGACCII	GCCGTCGTCG	CGGGCCTGAA	CTTCCGGGAT	GTCACGGTCG
55		CGCTCGGTGT	GGTCGCCGAT		TCGGCAGCGA		GTCGTCCTGG
		AGACCGGCCC				GGTCCTGGGG	
			ACCGGTCGCG			CGGCCGGATG	
	66721				CCGCGTTCGC		TACGGCCTGG
40	66781	TCGACCTGGC					GCGACCGGTG
	66841	TCGGCGCGGC	GGCCGTCCAG	ATOGOGOGGC	ATCTGGGGGG	GGAGGTGTAC	GCGACCACCA
		GCGCCGCGAA	GCGCCATCTG	GTGGACCTGG	ACGGAGCGCA	TCTGGCCGAT	TCCCGCAGCA
	66961	CCGCGTTCGC	CGACGCGTTC	CCGCCGGTCG	ATGTCGTGCT	CAACTCGCTC	ACCGGTGAAT
	67021	TCCTCGACGC	GTCCGTCGGC	CIGCTCGCGG	CGGGTGGCCG	GTTCATCGAG	ATGGGGAAGA
45	67081	CGGACATCCG	GCACGCCGTC	CAGCAGCCGT	TCGACCTGAT	GGACGCCGGC	CCCGACCGGA
	67141	TGCAGCGGAT	CATCGTCGAG	CIGCTCGGCC	TGTTCGCGCG	CGACGTGCTG	CACCCGCTGC
	67201	CGGTCCACGC	CTGGGACGTG	CGGCAGGCGC	GGGAGGCGTT	CGGCTGGATG	AGCAGCGGGC
	67261	GTCACACCGG	CAAGCTGGTG	CTGACGGTCC	CGCGGCCGCT	GGATCCCGAG	GGGGCCGTCG
~ O	67321	TOATCACCGG	CGGCTCCGGC	ACCOTOGCO	GCATCCTCGC	CCGCCACCTG	GGCCACCCCC
50	67381	ACACCTACCT	GCTCTCCCGC	ACCCCACCC	CCGACACCAC	CCCCGGCACC	CACCTCCCCT
	67441	GCGACGTCGG	CGACCCCCAC	CARCTCGCCA	CCACCCTCGC	CCGCATCCCC	CAACCCCTCA
	67501	CCGCCGTCTT	CCACACCGCC	GGAACCCTCG	ACGACGCCCT	GUTUGACAAC	CTCACCCCCG
	67561	ACCGCGTCGA	CACCGTCCTC	AAACCCAAGG	CCGACGCCGC	CIGGCACCIG	CACCGGCTCA
55	6/621	CCCGCGACAC	CGACCTCGCC	GCGTTCGTCG	TOTACTUCGU	CCACCCCCCC	CCCCAAGAGA
55	6/681	GCCCGGGGCA	GGGCAACTAC	G.CGCGGCGA	#CGCG11CC1	CATCTCCCCC	CACCECACCC
	67001	GCCGTGCGCA CGCTCACCGC	AGGGCTGCCC	CACCCCACTCCC	CCCACCCCAT	CUTATAGAGA	GCATTCCCC
	6706	CGCTCACCGC	GAAACTCACC	TOCCCCO	TOCACCOCA.	GACGCGTACC	CCGGDACCGG
	67001	TOGTOGTOGO	CACCACCCC	A.GCGGCTGT	FOUNCECOSC	CECCETCECC	CCGGMACCGG
60	6/921	GCGGTCGTCGC	GACGACCGTC	COCCACCO	- CCCCCCCCCC	- CGCCG1CGCG	CCGIIGCIEC
00	60041	AGCCCCTGGC	COTOCOMORM	00000000000	COCOCACOCA	GCAGCGGCGC	ATCATECAGE
	69101	AGCCCCTGGC	CCCCCACCC	GCCGCGCTACC	TCGCGTACGG	CCTGGGCGAC	CGCGTGGCGG
	68161	CGGACCGTCC	CTTCCCCCAC	- CTCGCTTTTCG	ATTCGCTGAC	CGCGGTCGAC	CTGCGCAATC
	68221	GGCTCGCGGC	CCACACCCCC	CTGCGGTTCC	CGACGACGCT	GGTGTTCAGC	CACCCGACGG
	00221	200120000	CORONCOSOS	0.0000100	55557.5551		

	68281	CGGAGGCGCT		CTGCTCGACC	TGATCGACGC	TCCCACCGCC	CGGATCGCCG
	68341	GGGAGTCCCC		A000000000	CCGTGGCGGC	CGCGCGGGAC	CAGGACGAGC
	68401	CGATCGCCAT	COTOGOGATG		TOCCCSSTGG	TGTGACGTCG	
_	68461	TGTGGCGGCT	COTTOAGTOD	GSCACCGACS	DGATTACCAC	GCCTCCTGAC	
5	68521	GGGAGGTCGA	CGCGCTGTAC	GACGCGGACC	030A030330	CGGCAAGGCG	
	68581	GGGGGGTTA	מינושטיים מינו בעיני	3.303 <i>an</i> 13.	TOGACGCGGC	STTCTTCGAC	
	68641	GUGAAGCGCT	CGGCATGGAC	GGGGAGGAAG		CGAAACGGCG	
	68701	TEGAGEGEGG	CCGGATCAGT	COGGCGTCGC	TCCGCGGGGGG	GGAGGTCGGC	
10	68761	STGCGGCCGC	GCAGGGCTAC	GGGCTGGGCG	CCGAGGAGAC	CGAGGGCCAC	•
10	68821	GIGGITTOCAC	GAGCCTGCTG	TOCGGACGGC	TGGCGTACGT	GCTCGGGCTG	
	68831	OGGTCACCGT	GGACACGGCG	TGCTCGTCGT	erergaráse.	GCTGCATCTG	
	68941	GGCTGCGCCT	GGGCGAGTGC	GAACTCGCTC	TGGCCC33AGG	GGTCTCCGTA GGCCGACGGG	
	69001		CGTGGAGTTC	TCCCGCCAGC	60000000000		CGCTGCAAGT
15	69061	CGTTCGGCGC		GGCACGACGT	GGTCCGAGGG ACACCGTGCT	CGTGGGCGTG	
13	69121 69191	AACGGCTCTC CCGTCACGTC		CGGCTCGGGC	TCACCCCCCCC	GAACGGGCTC	
	69241			TOCAACGGCC GCCGCGGCCG	GGCTGACCGG	CGCCGACGTG	
				CGGCTCGGCG	ACCCCCTCCA	GGCGGACGCG	
		CGTACGGGCA		GGACCGGTCT	GGCTGGGGCTC	GCTGAAGTCG	
20	69421	ATGCCACGGC	CGCGGCCGGT	GTCGCGGGGCG	TCATCAMGAT	GGTGCAGGCG	
ر) سر	69481		GCGGACGCTG	CATGTGGAGG	AGCCCTCGTC	CGCCGTCGAC	TGGAGCACCG
	69541		CCTGCTCGGC	TCCAACCGGC	CCTGGCGGA	CGACGAGCGT	CCGCGCCCGGG
	69601		CGCGTTCGGG	CTCAGCGGGA	CGAAGGGGGA		GAACAGCACC
	69661	GTCCGGCGCC		CAGCCGCCCC	0000000000		CAGCCGCTGC
25		CGTGGGTGCT			CGC1'903990	CCAGGGGGGC	-
		ACCACCTOGC			CGTTGGACAT	CGGGTACGCG	
		GCCGCGCCCA			TOGTOGOCAC	CACCCCGGAC	
		CCGCGCTCGA				AGTCGTCACC	GGGACCGCTC
	69961	AGGAGCGGCG			GCCAGGGCGC	CCAGCGCGCC	
30	70021	GCGAGCTCCA	CCGCCGGTTC	CCCGTCTTCG	CCGCCCCCTTG	GGACGAGGTC	TCCGACGCGT
	7008.	TOGGCAAGCA	CCTCAAGCAC	TECECCACGG	ACGTCTACCA	CGGCGAACAC	GGCGCTCTCG
	70141	CCCATGACAC	CCTGTACGCC	CAGGCCGGCC	TGTTCACGCT	CGAAGTGGCG	CTGCTGCGGC
	70201	TGCTGGAGCA	CTGGGGGGTG	CGGCCGGACG	TGCTCGTCGG	GCACTCCGTC	GGCGAGGTGA
	70261	CCGCGGCGTA	CGCGGCGGGG	GTGCTCACCC	TGGCGGACGC	GACGGAGTTG	ATCGTGGCCC
35	70321	GGGGGGGGGC	GCTGCGGGCG	CTGCCGCCCG	GGGCGATGCT	CGCCGTCGAC	GGAAGCCCGG
	70381	CGGAGGTCGG	CGCCCGCACG	GATCTGGACA	TOGCCGCGGT	CAACGGCCCG	TCCGCCGTGG
	70441	TGCTCGCCGG				GGAGTGGTCG	
	70501	GGCGCACGAA			CGTTCCACTC	CCGGCACGTC	
	70561		CCGTACGGTG		TOGOGTTOGG	CGCGGCGCGG	
40	70601	TGTOCACGAC	GACGGGCCGG	GACGCCSCGG		AACGCCCGCG	
	70881		resseessis			GGAGCTGGCC	
	70741	TCACCACGTT	CGTGGCCGTC	GGCCCCTCCG	GCTCCCTGGC	GTCGGCCGCG	GCGGAGAGCG
		CCGGGGAGGA					
15		CGGCGCTGAC					
45		TACIGGCCGG					
		GGCTGGCCCC					
		AGTCCGAGCC					
	71101	TCGGCGTCAC ACTCACTGGC	GGACCCCGCC	CMCCCCCAIG	T COMPAGE OFF	CCCARCCCCC	CTCGGTTTCG
50	71.01	CGGCGGCCGT	COMORMOCIC	CIGCGCMAC'	- Closcold	CACCCCCTTC	CTCCAGGACC
50	71201	GGATCGAGGC	CC.GIICGAU	CCCTTCCTCC	CGGCGCGCT	CRACCACCATTC	CCCACCGTGC
	71201	TOTOGOTOGT	CGACCAGATA	CASTCACCA	ACGCCCCCGGA	CATCGCGGCG	ACGCCGGCCC
		CGGAGCGTGC					
	71461	GATGAGCACC	GATACGCACG	AGGGLACGCC	GCCCGCCGGC	CGCTGCCCAT	TCGCGATCCA
55	71521	GGACGGTCAC	CCCCCATCC	TEGAGAGEGE	CACGGTGGGT	TOGTTOGACO	TGTTCGGCGT
55		CAAGCACTGG					
	71641	CAGCTCGGCC	GCGCCGTCCG	AGATGCTGCC	CGACCGGCGG	CCCGGCTGGT	TCTCCGGGAT
	71701	GGACTCACCG	GAGCACAACC	GCTACCGGCL	GAAGATOGOG	GGGGACTTCA	CACTGCGCGC
	71761	GGCGCGCAAG	CGGGAGGACT	TOGTOGOGGA	GGCCGCCGAC	GCCTGCCTGG	ACGACATCGA
60	71821	GGCCGCGGGA	CCCGGCACCG	ACCTCATCCC	CGGGTACGCC	AAGCGGCTGC	CCTCCCTCGT
55	71881	CATCAACGCG	CTGTACGGGC	TCACCCCTGA	GGAGGGGGCC	GTGCTGGAGG	CACGGATGCG
	71941	CGACATCACC	GGCTCGGCCG	ATCTGGACAG	CGTCAAGACG	CTGACCGACG	ACTTCTTCGG
	72001	GCACGCGCTG	CGGCTGGTCC	GCGCGAAGCG	TGACGAGCGG	GGCGAGGACC	TGCTGCACCG
	72061	GCTGGCCTCG	GCCGACGACG	GCGAGATCTC	GCTCAGCGAC	GACGAGGCGA	CGGGCGTGTT
	5 - 1	11110000000					

	20101	0.0.0.0.0.0.0.0.0.0.0.0.0.0.0.0.0.0.0.0.		CCChochom	2000222		
			CTGTTCGCCG				
	72131	CUCACTOCTC	AGCCACCCCG	AGUAGUAGGC	GGUGCTGCGC	GCGCGCCCGG	AGCTGGTCGA
	1 L L L L		GAGGAGATGC				
5			GACGTOGATG				
5	72361					CAGCCCGACA	
	72421		CTGGAGGGCA				
	72481		CGGGTGCTCA			TTGTTCGAGC	
	72541					GGGCTGTTCA	
1.()	72601		ACCTGGGGGG				
10	72661		TCGCGCACAT			TCCTCTACCG	
	72731		GCTACCTGCG				
		GTCGGCGCGA				TGGAGTGTCC	
		GTGCACGCCT	TCGAGCCCGC	GCCCGTGCCG	TTCGCGGGGGG	TGCGGGCGAA	CGTGACGCGG
	72901		CGGGCCAGGC				
15			ATCCCGACGC				
	73021	ACGGAGCTGT	TGCGCACGCT	CGGCCTCAAC	GGCGGCTACA	CCGCCGAGGA	CGTCGACACC
	73061	ATGCTCGCGC	AACTGCCCGA	CGTCAGCGAG			
	73141	GACGTCATCG	CGGAGCGCGG	TATOGAGGO :	AndGGGGGGG	TGAAGGTCGA	CGTGGAGAAG
			AGGTCTTCGC	CGGCCTCGAG	GACACOGACT	GGCCCCGTAT	CCGCCAGGTC
20	73261	GTCGCGGAGG	TCCACGACAT	CGACGGCGCG	CTCGAGGAGG	TOGTCACGOT	GCTCCGCGGC
	33321	CATGGCTTCA	CCGTGGTCGC	CGAGCAGGAA	COGCTGTTCG	CCGGCACGGG	CATCCACCAG
	33351	STOGGGGGGG	GGCGGGTGGC	CGGCTGAGCG	CCGTCGGGGC	CGCGGCCGTC	CGCACCGGCG
	73441	GCCGCGGTGC	GGACGGCGGC	TOAGDOGGGG	TOGGACAGTT	CCTTGGGCAG	TTGCTGACGG
	73501	COCCTCACCC	CCAGCTTGCG				
25	73561		GGCTGGCGAT				
	73621	CGCCGCTCCG	CCTCGGTCAG	CGATG.GATC	CGCTGCGCCC	GCGTCACGTC	CTGGGTGCCG
			AGGACTCCCC				
			GTGCGCGGCG				
	73901	CACGETTEGE	CCATGTCGGC	GAGGACGCGG	GCCAGCTCGT	ACTGGTCGCG	GCACATGATG
30			CGGCCTCGTC				
			GUGTCATCAC				
			GCCCCTCGTC				
			GGGCCAGGCC				
			CGTTGTACGC				
35			TGTGCAGTCC				
			CCGCCCGGTC				
			CGGCGGCCAT				
			CGGCGGCGGT				
			CCTCGTCGGC				
40			CGGCATCGGT				
			CCGTCGTGAC				
			AGCCGCGCAG				
			AAAACGAGGC				
			CGGGATAGAT				
45			TCGGGGCGGC				
			TCGCCAGCCA				
			CGGTGAGCAG				
			CGGCGGTGTC				
			ACTCCAGGTA				
50			CGGTGAACAG				
50			CGAGCACCTT				
			CACGCCGCTC				
			ACCGCCCTTC				
			CGGTGTCGAG				
55			CGGAAGCTCG				
55			CGAGCCGGTA				
			CCCGGATGTC				
			CCTGGGCCAC				
60			TCTGCGCCTC				
60			CCGCCCGGAA				
			CACGGGCCCG				
			CGTGGTGCAC				
			TGCGGGTGAG				
	/5901	TCGCACGATG	CCGTCAGCCG	GACCAGCTCC	GGTGTCCGGG	CGGCCAGCTC	GGGCTGGTCG

	75961	AGGAGCTGGC	CGAGCATGCC	GTACGGCAGG	GCCCGCTCCT	CCATGGAGCA	CACCGCGCGA
	76021	AGGGTGACGA	AGCCGGCCTT	39000003303	GCGTCGAGGA	STICGGICTT	GCCGCAGGCG
	76061	ATOSSCOOSS	TGACGGCGCC	GACGACGCC	36 333363 333	COGCTCGGGT	GAGCGCCCGG
_	~614!	TBBAGGGAAC	CGAACTCGTC	ATCCCCGGGGG	ATCAGGTCTS	GGGGAGATAA	GCGCGCTATC
5	7.5260	ACGAATGGAA	CTACCTCCCG	ADDSTDOTOS	AAACCCATAĞ	GCATCACATG	GCTTGTTGAT
	16261	CTGTACGGCT	GTGATTCAGC	07330333AT	GATGTGTAT	AGATIGGGAAG	ATGTGATCTA
	76311	3660037600	GTTCCCTCAG	GRGCCGRCCG	00000033331	CACCCGCCGT	ACCGCCTGGG
	76381	CCACCAGCIC	GGCGACCCGC	TCCTGGTGGT	CGACGAGGTA	GAAGTGCCCG	CCGGGGAAGA
	76441	CCTCCACCGT	GGTCGGCGCG	GTCGTGTGCC	CGGCCCAGGC	GTGGGCCTGC	TCCACCGTCG
10	76501	TOTTOGGATO	GTCGTCACCG	ATGCACACCG	TGATCGGCGT	CTCCAGCGGC	GGCGCGGGCT
	76561	CCCACCGGTA	CGTCTCCGCC	GCGTAGTAGT	COGOCOGCAA	CGGCGCCAGG	ATCAGCGCGC
	76621	GCATTTCGTC	GTCCGCCATC	ACATOGGOGO	TOGTCCCGCC	SAGGCCGATG	ACCGCCGCCA
	76681	GCAGCTCGTC	GTCGGACGCG	AGGTGGTCCT	GGTCGGCGCG	COGCTGCGAC	GGCGCCCGCC
	76741	GGCCCGAGAC	GATCAGGTGC	GUDACCGGGA	GCCGCTGGGC	CAGCTCGAAC	GCGAGTGTCG
15	76801	CGCCCATGCT	GTGGCCGAAC	AGCACCAGCG	GACGGTCCAG	CCCCGGCTTC	AACGCCTCGG
	76861	CCACGAGGCC	GGCGAGAACA	CGCAGGTCGC	GTACCGCCTC	CTCGTCGCGG	CGGTCCTGGC
	76921	GGCCGGGGGTA	CTGCACGGCG	TACACGTCCC	COACC 30360	GAGCGCACGG	GUUAGCGGAA
	76981	GGTAGAACGT	CGCCGATCCG	coggan" 33	GCAGCAGCAC	SACCOGTACC	GGGGCCTCGG
	77041	GOGTGGGGAA	GAACTGCCGC	AGCCAGAGTT	CCGAGCTCAC	CGCACCCCCT	CGGCCGCGAC
20	77101	CTGGGGAGCC	CGGAACCGGG	TGATCTCGGC	CAAGTGCTTC	TOCOGCATCT	CCGGGTCGGT
	77161	CACGCCCCAT	CCCTCCTCCG	GOGOCAGACA	GAGGACGCCG	ACTTTGCCGT	TGTGCACATT
	77221	GCGATGCACA	TCGCGCACCG	COGRECEGRE	GTCCTCCAGC	GGGTAGGTCA	CCGACAGCGT
	77281	CGGGTGCACC	ATCCCCTTGC	AGATCAGGCG	GTTCGCCTTC	CACGCCTCAC	GATAGTTCGC
	77341	GAAGTGGGTA	CCGATGATCC	GCTTCACGGA	CATCCACAGG	THOOGATTGT	CAAAGGCGTG
25	77401	CTCGTATCCC	GAGGTTGACG	CGCAGGTGAC	GATOGTOCCA	CCCCGACGTG	TCACGTIGAC
	77461	ACTCGCGCCG	AACGTCGCGC	GCCCCGGGTG	CTCGAACAUG	ATGTCGGGAT	CGTCACCGCC
	77521	GGTCAGCTCC	CGGATC				

Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given amino acid sequence of the invention. The native DNA sequence encoding the FK-520 PKS of *Streptomyces hygroscopicus* is shown herein merely to illustrate a preferred embodiment of the invention, and the present invention includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the invention. In similar fashion, a polypeptide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The present invention includes such polypeptides with alternate amino acid sequences, and the amino acid sequences shown merely illustrate preferred embodiments of the invention.

The recombinant nucleic acids, proteins, and peptides of the invention are many and diverse. To facilitate an understanding of the invention and the diverse compounds and methods provided thereby, the following general description of the FK-520 PKS genes and modules of the PKS proteins encoded thereby is provided. This general description is followed by a more detailed description of the various domains and modules of the FK-520 PKS contained in and encoded by the compounds of the invention. In this description, reference to a heterologous PKS refers to any PKS other than the FK-520 PKS. Unless otherwise indicated, reference to a PKS includes reference

to a portion of a PKS. Moreover, reference to a domain, module, or PKS includes reference to the nucleic acids encoding the same and vice-versa, because the methods and reagents of the invention provide or enable one to prepare proteins and the nucleic acids that encode them.

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The FK-520 PKS is composed of three proteins encoded by three genes designated fkbA, fkbB, and fkbC. The fkbA ORF encodes extender modules 7 - 10 of the PKS. The fkbB ORF encodes the loading module (the CoA ligase) and extender modules 1 - 4 of the PKS. The fkbC ORF encodes extender modules 5 - 6 of the PKS. The fkbP ORF encodes the NRPS that attaches the pipecolic acid and cyclizes the FK-520 polyketide.

The loading module of the FK-520 PKS includes a CoA ligase, an ER domain, and an ACP domain. The starter building block or unit for FK-520 is believed to be a dihvdroxycyclohexene carboxvlic acid, which is derived from shikimate. The recombinant DNA compounds of the invention that encode the loading module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of methods and in a variety of compounds. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 loading module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for the loading module of the heterologous PKS is replaced by the coding sequence for the FK-520 loading module, provides a novel PKS coding sequence. Examples of heterologous PKS coding sequences include the rapamycin, FK-506, rifamycin, and avermectin PKS coding sequences. In another embodiment, a DNA compound comprising a sequence that encodes the FK-520 loading module is inserted into a DNA compound that comprises the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the loading module coding sequence is utilized in conjunction with a heterologous coding sequence. In this embodiment, the invention provides, for example, either replacing the CoA ligase with a different CoA ligase, deleting the ER, or replacing the ER with a different ER. In addition, or alternatively, the ACP can be replaced by another ACP. In similar fashion, the corresponding domains in another loading or extender module can be replaced by one or more domains of the FK-520 PKS. The resulting heterologous loading module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide.

The first extender module of the FK-520 PKS includes a KS domain, an AT domain specific for methylmalonyl CoA. a DH domain, a KR domain, and an ACP domain. The recombinant DNA compounds of the invention that encode the first extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 first extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the first extender module of the FK-520 PKS or the latter is merely access to coding sequences for modules of the latter is movel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the first extender module of the FK-520 PKS is inserted into a DNA compound that comprises the remainder of the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

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In another embodiment, all or only a portion of the first extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2hydroxymalonyl CoA specific AT; deleting either the DH or KR or both; replacing the DH or KR or both with another DH or KR; and/or inserting an ER. In replacing or inserting KR, DH, and ER domains, it is often beneficial to replace the existing KR, DH, and ER domains with the complete set of domains desired from another module. Thus, if one desires to insert an ER domain, one may simply replace the existing KR and DH domains with a KR, DH, and ER set of domains from a module containing such domains. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a gene for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous first extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the first extender module of the FK-520 PKS.

In an illustrative embodiment of this aspect of the invention, the invention provides recombinant PKSs and recombinant DNA compounds and vectors that encode

such PKSs in which the KS domain of the first extender module has been inactivated. Such constructs are especially useful when placed in translational reading frame with the remaining modules and domains of an FK-520 or FK-520 derivative PKS. The utility of these constructs is that host cells expressing, or cell free extracts containing, the PKS encoded thereby can be fed or supplied with N-acyleysteamine thioesters of novel precursor molecules to prepare FK-520 derivatives. See U.S. patent application Serial No. 60/117,384, filed 27 Jan. 1999, and PCT patent publication Nos. US97/02358 and US99/03986, each of which is incorporated herein by reference.

The second extender module of the FK-520 PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, an inactive DH, and an ACP. The recombinant DNA compounds of the invention that encode the second extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 second extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the second extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the second extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, all or a portion of the second extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR and/or the inactive DH; replacing the KR with another KR; and/or inserting an active DH or an active DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous second extender module coding sequence can be utilized in conjunction with a coding sequence from a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding

domains in a module of a heterologous PKS can be replaced by one or more domains of the second extender module of the FK-520 PKS.

The third extender module of the FK-520 PKS includes a KS, an AT specific for malonyl CoA, a KR, an inactive DH, and an ACP. The recombinant DNA compounds of the invention that encode the third extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 third extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the third extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the third extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, all or a portion of the third extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR and/or the inactive DH; replacing the KR with another KR; and/or inserting an active DH or an active DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous third extender module coding sequence can be utilized in conjunction with a coding sequence from a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the third extender module of the FK-520 PKS.

The fourth extender module of the FK-520 PKS includes a KS, an AT that binds ethylmalonyl CoA, an inactive DH, and an ACP. The recombinant DNA compounds of the invention that encode the fourth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In

one embodiment, a DNA compound comprising a sequence that encodes the FK-520 fourth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the fourth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the fourth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the remainder of the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the fourth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the ethylmalonyl CoA specific AT with a malonyl CoA, methylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; and/or deleting the inactive DH, inserting a KR, a KR and an active DH, or a KR, an active DH, and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, a PKS for a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous fourth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the fourth extender module of the FK-520 PKS.

As illustrative examples, the present invention provides recombinant genes, vectors, and host cells that result from the conversion of the FK-506 PKS to an FK-520 PKS and vice-versa. In one embodiment, the invention provides a recombinant set of FK-506 PKS genes but in which the coding sequences for the fourth extender module or at least those for the AT domain in the fourth extender module have been replaced by those for the AT domain of the fourth extender module of the FK-520 PKS. This recombinant PKS can be used to produce FK-520 in recombinant host cells. In another embodiment, the invention provides a recombinant set of FK-520 PKS genes but in which the coding sequences for the fourth extender module or at least those for the AT domain in the fourth extender module have been replaced by those for the AT domain of

the fourth extender module of the FK-506 PKS. This recombinant PKS can be used to produce FK-506 in recombinant host cells.

Other examples of hybrid PKS enzymes of the invention include those in which the AT domain of module 4 has been replaced with a malonyl specific AT domain to provide a PKS that produces 21-desethyl-FK520 or with a methylmalonyl specific AT domain to provide a PKS that produces 21-desethyl-21-methyl-FK520. Another hybrid PKS of the invention is prepared by replacing the AT and inactive KR domain of FK-520 extender module 4 with a methylmalonyl specific AT and an active KR domain, such as, for example, from module 2 of the DEBS or oleandolide PKS enzymes, to produce 21-desethyl 21-methyl-22-desoxo-22-hydroxy-FK520. The compounds produced by these hybrid PKS enzymes are neurotrophins.

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The fifth extender module of the FK-520 PKS includes a KS, an AT that binds methylmalonyl CoA, a DH, a KR, and an ACP. The recombinant DNA compounds of the invention that encode the fifth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 fifth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the fifth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the fifth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the fifth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one or both of the DH and KR; replacing any one or both of the DH and KR with either a KR and/or DH; and/or inserting an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous fifth extender module coding sequence

can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the fifth extender module of the FK-520 PKS.

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In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the DH domain of the fifth extender module have been deleted or mutated to render the DH non-functional. In one such mutated gene, the KR and DH coding sequences are replaced with those encoding only a KR domain from another PKS gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-5000 flog that lacks the C-19 to C 20 double bond of FK-520 and has a C-20 hydroxyl group. Such analogs are preferred neurotrophins, because they have little or no immunosuppressant activity. This recombinant fifth extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this fifth extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (lacking the C-19 to C-20 double bond of FK-506 and having a C-20 hydroxyl group) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the DH domain of module 5 has been deleted or otherwise rendered inactive and thus produces this novel polyketide.

The sixth extender module of the FK-520 PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, a DH, an ER, and an ACP. The recombinant DNA compounds of the invention that encode the sixth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 sixth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the sixth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the sixth

extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the sixth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2hydroxymalonyl CoA specific AT; deleting any one, two, or all three of the KR, DH, and ER; and/or replacing any one, two, or all three of the KR. DH, and ER with another KR, 10 DH, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous sixth extender module coding 15 sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the sixth extender module of the FK-520 PKS.

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In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the DH and ER domains of the sixth extender module have been deleted or mutated to render them non-functional. In one such mutated gene, the KR, ER, and DH coding sequences are replaced with those encoding only a KR domain from another PKS gene. This can also be accomplished by simply replacing the coding sequences for extender module six with those for an extender module having a methylmalonyl specific AT and only a KR domain from a heterologous PKS gene, such as, for example, the coding sequences for extender module two encoded by the ervAl gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that has a C-18 hydroxyl group. Such analogs are preferred neurotrophins, because they have little or no immunosuppressant activity. This recombinant sixth extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this sixth extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-

506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (having a C-18 hydroxyl group) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the DH and ER domains of module 6 have been deleted or otherwise rendered inactive and thus produces this novel polyketide.

The seventh extender module of the FK-520 PKS includes a KS, an AT specific for 2-hydroxymalonyl CoA, a KR, a DH, an ER, and an ACP. The recombinant DNA compounds of the invention that encode the seventh extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA—ampound comprising a sequence that encodes the FK-520 seventh extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the seventh extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the seventh extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion or all of the seventh extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the 2-hydroxymalonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or malonyl CoA specific AT; deleting the KR, the DH, and/or the ER; and/or replacing the KR, DH, and/or ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous seventh extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the seventh extender module of the FK-520 PKS.

In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the AT domain of the seventh extender module has been replaced with those encoding an AT domain for malonyl, methylmalonyl, or ethylmalonyl CoA from another PKS gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that lacks the C-15 methoxy group, having instead a hydrogen, methyl, or ethyl group at that position, respectively. Such analogs are preferred, because they are more slowly metabolized than FK-520. This recombinant seventh extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this seventh extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (C-15desmethoxy) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the AT domain of module 7 has been replaced and thus produces this novel polyketide.

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In another illustrative embodiment, the present invention provides a hybrid PKS in which the AT and KR domains of module 7 of the FK-520 PKS are replaced by a methylmalonyl specific AT domain and an inactive KR domain, such as, for example, the AT and KR domains of extender module 6 of the rapamycin PKS. The resulting hybrid PKS produces 15-desmethoxy-15-methyl-16-oxo-FK-520, a neurotrophin compound.

The eighth extender module of the FK-520 PKS includes a KS, an AT specific for 2-hydroxymalonyl CoA, a KR, and an ACP. The recombinant DNA compounds of the invention that encode the eighth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 eighth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the eighth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the eighth extender

module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

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In another embodiment, a portion of the eighth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the 2-hydroxymalonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or malonyl CoA specific AT; deleting or replacing the KR; and/or inserting a DH or a DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous eighth extender module coding sequence can be utilized in conjunction with a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the eighth extender module of the FK-520 PKS.

In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the AT domain of the eighth extender module has been replaced with those encoding an AT domain for malonyl, methylmalonyl, or ethylmalonyl CoA from another PKS gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that lacks the C-13 methoxy group, having instead a hydrogen, methyl, or ethyl group at that position, respectively. Such analogs are preferred, because they are more slowly metabolized than FK-520. This recombinant eighth extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this eighth extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (C-13desmethoxy) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the AT domain of module 8 has been replaced and thus produces this novel polyketide.

The ninth extender module of the FK-520 PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, a DH, an ER, and an ACP. The recombinant DNA compounds of the invention that encode the ninth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 ninth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the ninth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the ninth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the ninth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT: deleting any one, two, or all three of the KR, DH, and ER; and/or replacing any one, two, or all three of the KR, DH, and ER with another KR, DH, and/or ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous ninth extender module coding sequence can be utilized in conjunction with a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the ninth extender module of the FK-520 PKS.

The tenth extender module of the FK-520 PKS includes a KS, an AT specific for malonyl CoA, and an ACP. The recombinant DNA compounds of the invention that encode the tenth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 tenth extender module is inserted into a DNA compound that comprises the coding sequence

for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the tenth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the tenth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

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In another embodiment, a portion or all of the tenth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; and/or inserting a KR, a KR and DH, or a KR, DH, and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous tenth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the tenth extender module of the FK-520 PKS.

The FK-520 polyketide precursor produced by the action of the tenth extender module of the PKS is then attached to pipecolic acid and cyclized to form FK-520. The enzyme FkbP is the NRPS like enzyme that catalyzes these reactions. FkbP also includes a thioesterase activity that cleaves the nascent FK-520 polyketide from the NRPS. The present invention provides recombinant DNA compounds that encode the *fkbP* gene and so provides recombinant methods for expressing the *fkbP* gene product in recombinant host cells. The recombinant *fkbP* genes of the invention include those in which the coding sequence for the adenylation domain has been mutated or replaced with coding sequences from other NRPS like enzymes so that the resulting recombinant FkbP incorporates a moiety other than pipecolic acid. For the construction of host cells that do not naturally produce pipecolic acid, the present invention provides recombinant DNA compounds that express the enzymes that catalyze at least some of the biosynthesis of pipecolic acid (see Nielsen *et al.*, 1991, *Biochem. 30*: 5789-96). The *fkbL* gene encodes a

homolog of RapL, a lysine cyclodeaminase responsible in part for producing the pipecolate unit added to the end of the polyketide chain. The *fkbB* and *fkbL* recombinant genes of the invention can be used in heterologous hosts to produce compounds such as FK-520 or, in conjunction with other PKS or NRPS genes, to produce known or novel polyketides and non-ribosmal peptides.

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The present invention also provides recombinant DNA compounds that encode the P450 oxidase and methyltransferase genes involved in the biosynthesis of FK-520. Figure 2 shows the various sites on the FK-520 polyketide core structure at which these enzymes act. By providing these genes in recombinant form, the present invention provides recombinant host cells that can produce FK-520. This is accomplished by introducing the recombinant PKS, P450 oxidase, and methyltransferase genes into a heterologous host cell. In a preferred embodiment, the heterologous host cell is *Streptomyces coelicolor* CH999 or *Streptomyces lividans* K4-114, as described in U.S. Patent No. 5,830,750 and U.S. patent application Serial Nos. 08/828,898, filed 31 Mar. 1997, and 09/181,833, filed 28 Oct. 1998, each of which is incorporated herein by reference. In addition, by providing recombinant host cells that express only a subset of these genes, the present invention provides methods for making FK-520 precursor compounds not readily obtainable by other means.

In a related aspect, the present invention provides recombinant DNA compounds and vectors that are useful in generating, by homologous recombination, recombinant host cells that produce FK-520 precursor compounds. In this aspect of the invention, a native host cell that produces FK-520 is transformed with a vector (such as an SCP2* derived vector for *Streptomyces* host cells) that encodes one or more disrupted genes (i.e., a hydroxylase, a methyltransferase, or both) or merely flanking regions from those genes. When the vector integrates by homologous recombination, the native, functional gene is deleted or replaced by the non-functional recombinant gene, and the resulting host cell thus produces an FK-520 precursor. Such host cells can also be complemented by introduction of a modified form of the deleted or mutated non-functional gene to produce a novel compound.

In one important embodiment, the present invention provides a hybrid PKS and the corresponding recombinant DNA compounds that encode those hybrid PKS enzymes. For purposes of the present invention a hybrid PKS is a recombinant PKS that comprises all or part of one or more modules and thioesterase/cyclase domain of a first PKS and all or part of one or more modules, loading module, and thioesterase/cyclase

domain of a second PKS. In one preferred embodiment, the first PKS is all or part of the FK-520 PKS, and the second PKS is only a portion or all of a non-FK-520 PKS.

One example of the preferred embodiment is an FK-520 PKS in which the AT domain of module 8, which specifies a hydroxymalonyl CoA and from which the C-13 methoxy group of FK-520 is derived, is replaced by an AT domain that specifies a malonyl, methylmalonyl, or ethylmalonyl CoA. Examples of such replacement AT domains include the AT domains from modules 3, 12, and 13 of the rapaymycin PKS and from modules 1 and 2 of the erythromycin PKS. Such replacements, conducted at the level of the gene for the PKS, are illustrated in the examples below. Another illustrative example of such a hybrid PKS includes an FK-520 PKS in which the natural loading module has been replaced with a loading module of another PKS. Another example of such a hybrid PKS is an FK-520 PKS in which the AT domain of module three is replaced with an AT domain that binds methylmalonyl CoA.

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In another preferred embodiment, the first PKS is most but not all of a non-FK-520 PKS, and the second PKS is only a portion or all of the FK-520 PKS. An illustrative example of such a hybrid PKS includes an erythromycin PKS in which an AT specific for methylmalonyl CoA is replaced with an AT from the FK-520 PKS specfic for malonyl CoA.

Those of skill in the art will recognize that all or part of either the first or second PKS in a hybrid PKS of the invention need not be isolated from a naturally occurring source. For example, only a small portion of an AT domain determines its specificity. See U.S. provisional patent application Serial No. 60/091,526, incorporated herein by reference. The state of the art in DNA synthesis allows the artisan to construct *de novo* DNA compounds of size sufficient to construct a useful portion of a PKS module or domain. For purposes of the present invention, such synthetic DNA compounds are deemed to be a portion of a PKS.

Thus, the hybrid modules of the invention are incorporated into a PKS to provide a hybrid PKS of the invention. A hybrid PKS of the invention can result not only:

- (i) from fusions of heterologous domain (where heterologous means the domains in that module are from at least two different naturally occurring modules) coding sequences to produce a hybrid module coding sequence contained in a PKS gene whose product is incorporated into a PKS, but also:
 - (ii) from fusions of heterologous module (where heterologous module means two modules are adjacent to one another that are not adjacent to one another in naturally

occurring PKS enzymes) coding sequences to produce a hybrid coding sequence contained in a PKS gene whose product is incorporated into a PKS,

(iii) from expression of one or more FK-520 PKS genes with one or more non-FK-520 PKS genes, including both naturally occurring and recombinant non-FK-520 PKS genes, and

(iv) from combinations of the foregoing.

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Various hybrid PKSs of the invention illustrating these various alternatives are described herein.

Examples of the production of a hybrid PKS by co-expression of PKS genes from the FK-520 PKS and another non-FK-520 r . S include hybrid PKS enzymes produced by coexpression of FK-520 and rapamycin PKS genes. Preferably, such hybrid PKS enzymes are produced in recombinant *Streptomyces* host cells that produce FK-520 or FK-506 but have been mutated to inactivate the gene whose function is to be replaced by the rapamycin PKS gene introduced to produce the hybrid PKS. Particular examples include (i) replacement of the *fkbC* gene with the *rapB* gene; and (ii) replacement of the *fkbA* gene with the *rapC* gene. The latter hybrid PKS produces 13,15-didesmethoxy-FK-520, if the host cell is an FK-520 producing host cell, and 13,15-didesmethoxy-FK-506, if the host cell is an FK-506 producing host cell. The compounds produced by these hybrid PKS enzymes are immunosuppressants and neurotrophins but can be readily modified to act only as neurotrophins, as described in Example 6, below.

Other illustrative hybrid PKS enzymes of the invention are prepared by replacing the *fkbA* gene of an FK-520 or FK-506 producing host cell with a hybrid *fkbA* gene in which: (a) the extender module 8 through 10, inclusive, coding sequences have been replaced by the coding sequences for extender modules 12 to 14, inclusive, of the rapamycin PKS; and (b) the module 8 coding sequences have been replaced by the module 8 coding sequence of the rifamycin PKS. When expressed with the other, naturally occurring FK-520 or FK-506 PKS genes and the genes of the modification enzymes, the resulting hybrid PKS enzymes produce, respectively, (a) 13-desmethoxy-FK-520 or 13-desmethoxy-FK-506; and (b) 13-desmethoxy-13-methyl-FK-520 or 13-desmethoxy-13-methyl-FK-506. In a preferred embodiment, these recombinant PKS genes of the invention are introduced into the producing host cell by a vector such as pHU204, which is a plamsid pRM5 derivative that has the well-characterized SCP2* replicon, the *colE1* replicon, the *tsr* and *bla* resistance genes, and a *cos* site. This vector can be used to introduce the recombinant *fkbA* replacement gene in an FK-520 or FK-506 producing host cell (or a host cell derived therefrom in which the endogenous fkbA

gene has either been rendered inactive by mutation, deletion or homologous recombination with the gene that replaces it) to produce the desired hybrid PKS.

In constructing hybrid PKSs of the invention, certain general methods may be helpful. For example, it is often beneficial to retain the framework of the module to be altered to make the hybrid PKS. Thus, if one desires to add DH and ER functionalities to a module, it is often preferred to replace the KR domain of the original module with a KR, DH, and ER domain-containing segment from another module, instead of merely inserting DH and ER domains. One can alter the stereochemical specificity of a module by replacement of the KS domain with a KS domain from a module that specifies a different stereochemistry. See Lau et al., 12... Dissecting the role of acyltransferase domains of modular polyketide synthases in the choice and stereochemical fate of extender units," Biochemistry 38(5):1643-1651, incorporated herein by reference. Stereochemistry can also be changed by changing the KR domain. Also, one can alter the specificity of an AT domain by changing only a small segment of the domain. See Lau et al., supra. One can also take advantage of known linker regions in PKS proteins to link modules from two different PKSs to create a hybrid PKS. See Gokhale et al., 16 Apr. 1999, "Dissecting and Exploiting Intermodular Communication in Polyketide Synthases," Science 284: 482-485, incorporated herein by reference.

The following Table lists references describing illustrative PKS genes and corresponding enzymes that can be utilized in the construction of the recombinant PKSs and the corresponding DNA compounds that encode them of the invention. Also presented are various references describing tailoring enzymes and corresponding genes that can be employed in accordance with the methods of the present invention.

Avermectin

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U.S. Pat. No. 5,252,474 to Merck.

MacNeil et al., 1993, Industrial Microorganisms: Basic and Applied Molecular Genetics, Baltz, Hegeman, & Skatrud, eds. (ASM), pp. 245-256, A Comparison of the Genes Encoding the Polyketide Synthases for Avermectin, Erythromycin, and Nemadectin.

MacNeil et al., 1992, Gene 115: 119-125, Complex Organization of the Streptomyces avermitilis genes encoding the avermectin polyketide synthase.

Ikeda *et al.*, Aug. 1999, Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*, *Proc. Natl. Acad. Sci. USA 96*: 9509-9514.

Candicidin (FR008)

Hu et al., 1994, Mol. Microbiol. 14: 163-172.

Epothilone

U.S. Pat. App. Serial No. 60'130,560, filed 22 April 1999.

Erythromycin

5 PCT Pub. No. 93/13663 to Abbott.

US Pat. No. 5,824,513 to Abbott.

Donadio et al., 1991, Science 252:675-9.

Cortes et al., 8 Nov. 1990, Nature 348:176-8, An unusually large multifunctional polypeptide in the erythromycin producing polyketide synthase of Saccharopolyspora erythraea.

Glycosylation Enzymes

PCT Pat. App. Pub. No. 97/23630 to Abbott.

FK-506

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Motamedi *et al.*, 1998, The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK-506, *Eur. J. biochem.* 256: 528-534.

Motamedi *et al.*, 1997, Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK-506, *Eur. J. Biochem. 244*: 74-80.

Methyltransferase

US 5,264,355, issued 23 Nov. 1993, Methylating enzyme from *Streptomyces* MA6858. 31-O-desmethyl-FK-506 methyltransferase.

Motamedi *et al.*, 1996, Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK-506 and FK-520, *J. Bacteriol.* 178: 5243-5248.

25 Streptomyces hygroscopicus

U.S. patent application Serial No. 09/154,083, filed 16 Sep. 1998.

Lovastatin

U.S. Pat. No. 5,744,350 to Merck.

Narbomycin

30 U.S. patent application Serial No. 60/107,093, filed 5 Nov. 1998, and Serial No. 60/120,254, filed 16 Feb. 1999.

Nemadectin

MacNeil et al., 1993, supra.

Niddamycin

Kakavas et al., 1997, Identification and characterization of the niddamycin polyketide synthase genes from *Streptomyces caelestis*, *J. Bacteriol*. 179: 7515-7522.

Oleandomycin

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Swan et al., 1994, Characterisation of a *Streptomyces antibioticus* gene encoding a type I polyketide synthase which has an unusual coding sequence, *Mol. Gen. Genet.* 242: 358-362.

U.S. patent application Serial No. 60/120,254, filed 16 Feb. 1999.

Olano et al., 1998, Analysis of a Streptomyces antibioticus chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring. Mol. Gen. Genet. 259(3): 299-308.

Picromycin

PCT patent application US99/15047, filed 2 Jul. 1999.

Xue et al., 1998, Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the pikC-encoded cytochrome P450 in Streptomyces venezuelae, Chemistry & Biology 5(11): 661-667.

Xue et al., Oct. 1998, A gene cluster for macrolide antibiotic biosynthesis in Streptomyces venezuelae: Architecture of metabolic diversity, Proc. Natl. Acad. Sci. USA 95: 12111 12116.

20 Platenolide

EP Pat. App. Pub. No. 791,656 to Lilly.

Rapamycin

Schwecke *et al.*, Aug. 1995, The biosynthetic gene cluster for the polyketide rapamycin, *Proc. Natl. Acad. Sci. USA 92*:7839-7843.

Aparicio et al., 1996, Organization of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: analysis of the enzymatic domains in the modular polyketide synthase, *Gene 169*: 9-16.

Rifamycin

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August et al., 13 Feb. 1998, Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the rif biosynthetic gene cluster of Amycolatopsis mediterranei S669, Chemistry & Biology, 5(2): 69-79.

Sorangium PKS

U.S. patent application Serial No. 09/144,085, filed 31 Aug. 1998.

Soraphen

35 U.S. Pat. No. 5,716,849 to Novartis.

Schupp et al., 1995, J. Bacteriology 177: 3673-3679. A Sorangium cellulosum (Myxobacterium) Gene Cluster for the Biosynthesis of the Macrolide Antibiotic Soraphen A: Cloning, Characterization, and Homology to Polyketide Synthase Genes from Actinomycetes.

5 Spiramycin

U.S. Pat. No. 5,098,837 to Lilly.

Activator Gene

U.S. Pat. No. 5,514,544 to Lilly.

Tylosin

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EP Pub. No. 791,655 to Lilly.

U.S. Pat. No. 5,876,991 to Lilly.

Kuhstoss *et al.*, 1996, *Gene 183*:231-6., Production of a novel polyketide through the construction of a hybrid polyketide synthase.

Tailoring enzymes

Merson-Davies and Cundliffe, 1994, *Mol. Microbiol. 13*: 349-355. Analysis of five tylosin biosynthetic genes from the *tylBA* region of the *Streptomyces fradiae* genome.

As the above Table illustrates, there are a wide variety of polyketide synthase genes that serve as readily available sources of DNA and sequence information for use in constructing the hybrid PKS-encoding DNA compounds of the invention. Methods for constructing hybrid PKS-encoding DNA compounds are described without reference to the FK-520 PKS in PCT patent publication No. 98/51695; U.S. Patent Nos. 5,672,491 and 5,712,146 and U.S. patent application Serial Nos. 09/073,538, filed 6 May 1998, and 09/141,908, filed 28 Aug 1998, each of which is incorporated herein by reference.

The hybrid PKS-encoding DNA compounds of the invention can be and often are hybrids of more than two PKS genes. Moreover, there are often two or more modules in the hybrid PKS in which all or part of the module is derived from a second (or third) PKS. Thus, as one illustrative example, the present invention provides a hybrid FK-520 PKS that contains the naturally occurring loading module and FkbP as well as modules one, two, four, six, seven, and eight, nine, and ten of the FK-520 PKS and further contains hybrid or heterologous modules three and five. Hybrid or heterologous module three contains an AT domain that is specific of methylmalonyl CoA and can be derived for example, from the erythromycin or rapamycin PKS genes. Hybrid or heterologous module five contains an AT domain that is specific for malonyl CoA and can be derived for example, from the picromycin or rapamycin PKS genes.

While an important embodiment of the present invention relates to hybrid PKS enzymes and corresponding genes, the present invention also provides recombinant FK-520 PKS genes in which there is no second PKS gene sequence present but which differ from the FK-520 PKS gene by one or more deletions. The deletions can encompass one or more modules and/or can be limited to a partial deletion within one or more modules. When a deletion encompasses an entire module, the resulting FK-520 derivative is at least two carbons shorter than the gene from which it was derived. When a deletion is within a module, the deletion typically encompasses a KR, DH, or ER domain, or both DH and ER domains, or both KR and DH domains, or all three KR, DH, and ER domains.

To construct a hybrid PKS or FK-520 derivative PKS gene of the invention, one can employ a technique, described in PCT Pub. No. 98/27203 and U.S. patent application Serial No. 08/989,332, filed 11 Dec. 1997, each of which is incorporated herein by reference, in which the large PKS gene is divided into two or more, typically three, segments, and each segment is placed on a separate expression vector. In this manner, each of the segments of the gene can be altered, and various altered segments can be combined in a single host cell to provide a recombinant PKS gene of the invention. This technique makes more efficient the construction of large libraries of recombinant PKS genes, vectors for expressing those genes, and host cells comprising those vectors.

Thus, in one important embodiment, the recombinant DNA compounds of the invention are expression vectors. As used herein, the term expression vector refers to any nucleic acid that can be introduced into a host cell or cell-free transcription and translation medium. An expression vector can be maintained stably or transiently in a cell, whether as part of the chromosomal or other DNA in the cell or in any cellular compartment, such as a replicating vector in the cytoplasm. An expression vector also comprises a gene that serves to produce RNA that is translated into a polypeptide in the cell or cell extract. Furthermore, expression vectors typically contain additional functional elements, such as resistance-conferring genes to act as selectable markers.

The various components of an expression vector can vary widely, depending on the intended use of the vector. In particular, the components depend on the host cell(s) in which the vector will be used or is intended to function. Vector components for expression and maintenance of vectors in *E. coli* are widely known and commercially available, as are vector components for other commonly used organisms, such as yeast cells and *Streptomyces* cells.

In a preferred embodiment, the expression vectors of the invention are used to construct recombinant *Streptomyces* host cells that express a recombinant PKS of the invention. Preferred *Streptomyces* host cell-vector combinations of the invention include *S. coelicolor* CH999 and *S. lividans* K4-114 host cells, which do not produce actinorhodin, and expression vectors derived from the pRM1 and pRM5 vectors, as described in U.S. Patent No. 5,830,750 and U.S. patent application Serial Nos. 08/828,898, filed 31 Mar. 1997, and 09-181,833, filed 28 Oct. 1998, each of which is incorporated herein by reference.

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The present invention provides a wide variety of expression vectors for use in 10 Streptom: 3. For replicating vectors, the origin of replication can be, for example and without limitation, a low copy number vector, such as SCP2* (see Hopwood et al., Genetic Manipulation of Streptomyces: A Laboratory manual (The John Innes Foundation, Norwich, U.K., 1985); Lydiate et al., 1985, Gene 35: 223-235; and Kieser and Melton, 1988, Gene 65: 83-91, each of which is incorporated herein by reference), 15 SLP1.2 (Thompson et al., 1982, Gene 20: 51-62, incorporated herein by reference), and SG5(ts) (Muth et al., 1989, Mol. Gen. Genet. 219: 341-348, and Bierman et al., 1992, Gene 116: 43-49, each of which is incorporated herein by reference). or a high copy number vector, such as pIJ101 and pJV1 (see Katz et al., 1983, J. Gen. Microbiol. 129: 2703-2714; Vara et al., 1989, J. Bacteriol. 171: 5782-5781; and Servin-Gonzalez, 1993, Plasmid 30: 131-140, each of which is incorporated herein by reference). Generally, 20 however, high copy number vectors are not preferred for expression of genes contained on large segments of DNA. For non-replicating and integrating vectors, it is useful to include at least an E. coli origin of replication, such as from pUC, p1P, p1I, and pBR. For phage based vectors, the phages phiC31 and KC515 can be employed (see Hopwood 25 et al., supra).

Typically, the expression vector will comprise one or more marker genes by which host cells containing the vector can be identified and/or selected. Useful antibiotic resistance conferring genes for use in *Streptomyces* host cells include the *ermE* (confers resistance to erythromycin and other macrolides and lincomycin), *tsr* (confers resistance to thiostrepton), *aadA* (confers resistance to spectinomycin and streptomycin), *aacC4* (confers resistance to apramycin, kanamycin, gentamicin, geneticin (G418), and neomycin), *hyg* (confers resistance to hygromycin), and *vph* (confers resistance to viomycin) resistance conferring genes.

The recombinant PKS gene on the vector will be under the control of a promoter, typically with an attendant ribosome binding site sequence. The present invention

provides the endogenous promoters of the FK-520 PKS and related biosynthetic genes in recombinant form, and these promoters are preferred for use in the native hosts and in heterologous hosts in which the promoters function. A preferred promoter of the invention is the *fkbO* gene promoter, comprised in a sequence of about 270 bp between the start of the open reading frames of the *fkbO* and *fkbB* genes. The *fkbO* promoter is believed to be bi-directional in that it promotes transcription of the genes *fkbO*, *fkbP*, and *fkbA* in one direction and *fkbB*, *fkbC*, and *fkbL* in the other. Thus, in one aspect, the present invention provides a recombinant expression vector comprising the promoter of the *fkbO* gene of an FK-520 producing organism positioned to transcribe a gene other than *fkbO* gene of mbodiment the transcribed gene is a gene that encodes a protein comprised in a hybrid PKS.

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Heterologous promoters can also be employed and are preferred for use in host cells in which the endogenous FK-520 PKS gene promoters do not function or function. poorly. A preferred heterologous promoter is the actl promoter and its attendant activator gene actII-ORF4, which is provided in the pRM1 and pRM5 expression vectors, supra. This promoter is activated in the stationary phase of growth when secondary metabolites are normally synthesized. Other useful Streptomyces promoters include without limitation those from the ermE gene and the melC1 gene, which act constitutively, and the tipA gene and the merA gene, which can be induced at any growth stage. In addition, the T7 RNA polymerase system has been transferred to Streptomyces and can be employed in the vectors and host cells of the invention. In this system, the coding sequence for the T7 RNA polymerase is inserted into a neutral site of the chromosome or in a vector under the control of the inducible merA promoter, and the gene of interest is placed under the control of the T7 promoter. As noted above, one or more activator genes can also be employed to enhance the activity of a promoter. Activator genes in addition to the actII-ORF4 gene discussed above include dnrI, redD, and ptpA genes (see U.S. patent application Serial No. 09/181,833, supra) to activate promoters under their control.

In addition to providing recombinant DNA compounds that encode the FK-520 PKS, the present invention also provides DNA compounds that encode the ethylmalonyl CoA and 2-hydroxymalonyl CoA utilized in the synthesis of FK-520. Thus, the present invention also provides recombinant host cells that express the genes required for the biosynthesis of ethylmalonyl CoA and 2-hydroxymalonyl CoA. Figures 3 and 4 show the

location of these genes on the cosmids of the invention and the biosynthetic pathway that produces ethylmalonyl Co.A.

For 2-hydroxymalonyl CoA biosynthesis, the *fkbH*, *fkbI*, *fkbJ*, and *fkbK* genes are sufficient to confer this ability on *Streptomcyces* host cells. For conversion of 2-

hydroxymalonyl to 2-methoxymalonyl, the *fkbG* gene is also employed. While the complete coding sequence for *fkbH* is provided on the cosmids of the invention, the sequence for this gene provided herein may be missing a T residue, based on a comparison made with a similar gene cloned from the ansamitocin gene cluster by Dr. H. Floss. Where the sequence herein shows one T, there may be two, resulting in an

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extension of the *fkbH* reading frame to encode the amino acid sequence:

MTIVKCLVWDLDNTLWRGTVLEDDEVVLTDEIREVITTLDDRGILQAVASKNDH

DLAWERLERLGVAEYFVLARIGWGPKSQSVREIATELNFAPTTIAFIDDQPAERA

EVAFHLPEVRCYPAEQAATLLSLPEFSPPVSTVDSRRRRLMYQAGFARDQAREA

YSGPDEDFLRSLDLSMTIAPAGEEELSRVEELTLRTSQMNATGVHYSDADLRAL

LTDPAHEVLVVTMGDRFGPHGAVGIILLEKKPSTWHLKLLATSCRVVSFGAGAT ILNWLTDQGARAGAHLVADFRRTDRNRMMEIAYRFAGFADSDCPCVSEVAGAS AAGVERLHLEPSARPAPTTLTLTAADIAPVTVSAAG.

For ethylmalonyl CoA biosynthesis, one requires only a crotonyl CoA reductase, which can be supplied by the host cell but can also be supplied by recombinant expression of the *fkbS* gene of the present invention. To increase yield of ethylmalonyl CoA, one can also express the *fkbE* and *fkbU* genes as well. While such production can be achieved using only the recombinant genes above, one can also achieve such production by placing into the recombinant host cell a large segment of the DNA provided by the cosmids of the invention. Thus, for 2-hydroxymalonyl and 2-methoxymalonyl CoA biosynthesis, one can simply provide the cells with the segment of DNA located on the left side of the FK-520 PKS genes shown in Figure 1. For ethylmalonyl CoA biosynthesis, one can simply provide the cells with the segment of DNA located on the right side of the FK-520 PKS genes shown in Figure 1 or, alternatively, both the right and left segments of DNA.

The recombinant DNA expression vectors that encode these genes can be used to construct recombinant host cells that can make these important polyketide building blocks from cells that otherwise are unable to produce them. For example, *Streptomyces coelicolor* and *Streptomyces lividans* do not synthesisze ethylmalonyl CoA or 2-hydroxymalonyl CoA. The invention provides methods and vectors for constructing recombinant *Streptomyces coelicolor* and *Streptomyces lividans* that are able to

synthesize either or both ethylmalonyl CoA and 2-hydroxymalonyl CoA. These host cells are thus able to make polyketides, those requiring these substrates, that cannot otherwise be made in such cells.

In a preferred embodiment, the present invention provides recombinant

Streptomyces host cells, such as S. coelicolor and S. lividans, that have been transformed with a recombinant vector of the invention that codes for the expression of the ethylmalonyl CoA biosynthetic genes. The resulting host cells produce ethylmalonyl CoA and so are preferred host cells for the production of polyketides produced by PKS enzymes that comprise one or more AT domains specific for ethylmalonyl CoA.

Illustrative PKS enzymes of this type include the FK-520 PKS and a recombinant PKS in which one or more AT domains is specific for ethylmalonyl CoA.

In a related embodiment, the present invention provides *Streptomyces* host cells in which one or more of the ethylmalonyl or 2-hydroxymalonyl biosynthetic genes have been deleted by homologous recombination or rendered inactive by mutation. For example, deletion or inactivation of the *fkbG* gene can prevent formation of the methoxyl groups at C-13 and C-15 of FK-520 (or, in the corresponding FK-506 producing cell, FK-506), leading to the production of 13,15-didesmethoxy-13,15-dihydroxy-FK-520 (or, in the corresponding FK-506 producing cell, 13,15-didesmethoxy-13,15-dihydroxy-FK-506). If the *fkbG* gene product acts on 2-hydroxymalonyl and the resulting 2-methoxymalonyl substrate is required for incorporation by the PKS, the AT domains of modules 7 and 8 may bind malonyl CoA and methylmalonyl CoA. Such incorporation results in the production of a mixture of polyketides in which the methoxy groups at C-13 and C-15 of FK-520 (or FK-506) are replaced by either hydrogen or methyl.

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This possibility of non-specific binding results from the construction of a hybrid PKS of the invention in which the AT domain of module 8 of the FK-520 PKS replaced the AT domain of module 6 of DEBS. The resulting PKS produced, in *Streptomyces lividans*, 6-dEB and 2-desmethyl-6-dEB, indicating that the AT domain of module 8 of the FK-520 PKS could bind malonyl CoA and methylmalonyl CoA substrates. Thus, one could possibly also prepare the 13,15-didesmethoxy-FK-520 and corresponding FK-506 compounds of the invention by deleting or otherwise inactivating one or more or all of the genes required for 2-hydroxymalonyl CoA biosynthesis, i.e., the *fkbH*, *fkbI*, *fkbJ*, and *fkbK* genes. In any event, the deletion or inactivation of one or more biosynthetic genes required for ethylmalonyl and/or 2-hydroxymalonyl production prevents the formation of polyketides requiring ethylmalonyl and/or 2-hydroxymalonyl for biosynthesis, and the

resulting host cells are thus preferred for production of polyketides that do not require the same.

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The host cells of the invention can be grown and fermented under conditions known in the art for other purposes to produce the compounds of the invention. See, e.g., U.S. Patent Nos. 5,194,378; 5,116,756; and 5,494,820, incorporated herein by reference, for suitable fermentation processes. The compounds of the invention can be isolated from the fermentation broths of these cultured cells and purified by standard procedures. Preferred compounds of the invention include the following compounds: 13-desmethoxy-FK-506; 13-desmethoxy-FK-520; 13,15-didesmethoxy-FK-506; 13-desmethoxy-FK-506; 13-desmethoxy-18-hydroxy-FK-506; and 13,15-didesmethoxy-18-hydroxy-FK-520; 13,15-didesmethoxy-18-hydroxy-FK-520. These compounds can be further modified as described for tacrolimus and FK-520 in U.S. Patent Nos. 5,225,403; 5,189,042; 5,164,495; 5,068,323; 4,980,466; and 4,920,218, incorporated herein by reference.

15 Other compounds of the invention are shown in Figure S. Parts A and B. In Figure 8, Part A, illustrative C-32-substituted compounds of the invention are shown in two columns under the heading R. The substituted compounds are preferred for topical administration and are applied to the dermis for treatment of conditions such as psoriasis. In Figure 8, Part B, illustrative reaction schemes for making the compounds shown in 20 Figure 8. Part A, are provided. In the upper scheme in Figure 8, Part B, the C-32 substitution is a tetrazole moiety, illustrative of the groups shown in the left column under R in Figure 8, Part A. In the lower scheme in Figure 8, Part B, the C-32 substitution is a disubstituted amino group, where R₃ and R₄ can be any group similar to the illustrative groups shown attached to the amine in the right column under R in Figure 25 8, Part A. While Figure 8 shows the C-32-substituted compounds in which the C-15methoxy is present, the invention includes these C-32-substituted compounds in which C-15 is ethyl, methyl, or hydrogen. Also, while C-21 is shown as substituted with ethyl or allyl, the compounds of the invention includes the C-32-substituted compounds in which C-21 is substituted with hydrogen or methyl.

To make these C-32-substituted compounds, Figure 8, Part B, provides illustrative reaction schemes. Thus, a selective reaction of the starting compound (see Figure 8, Part B, for an illustrative starting compound) with trifluoromethanesulfonic anhydride in the presence of a base yields the C-32 O-triflate derivative, as shown in the upper scheme of Figure 8, Part B. Displacement of the triflate with 1H-tetrazole or triazole derivatives provides the C-32 tetrazole or teiazole derivative. As shown in the

lower scheme of Figure 8, Part B, reacting the starting compound with pnitrophenylchloroformate yields the corresponding carbonate, which, upon displacement with an amino compound, provides the corresponding carbamate derivative.

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The compounds can be readily formulated to provide the pharmaceutical compositions of the invention. The pharmaceutical compositions of the invention can be used in the form of a pharmaceutical preparation, for example, in solid, semisolid, or liquid form. This preparation contains one or more of the compounds of the invention as an active ingredient in admixture with an organic or inorganic carrier or excipient suitable for external, enteral, or parenteral application. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. Suitable formulation processes and compositions for the compounds of the present invention are described with respect to tacrolimus in U.S. Patent Nos. 5,939,427; 5,922,729; 5,385,907; 5,338,684; and 5,260,301, incorporated herein by reference. Many of the compounds of the invention contain one or more chiral centers, and all of the stereoisomers are included within the scope of the invention, as pure compounds as well as mixtures of stereoisomers. Thus the compounds of the invention may be supplied as a mixture of stereoisomers in any proportion.

The carriers which can be used include water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, talc, corn starch, keratin, colloidal silica, potato starch, urea, and other carriers suitable for use in manufacturing preparations, in solid, semi-solid, or liquified form. In addition, auxiliary stabilizing, thickening, and coloring agents and perfumes may be used. For example, the compounds of the invention may be utilized with hydroxypropyl methylcellulose essentially as described in U.S. Patent No. 4,916,138, incorporated herein by reference, or with a surfactant essentially as described in EPO patent publication No. 428,169, incorporated herein by reference.

Oral dosage forms may be prepared essentially as described by Hondo *et al.*, 1987, *Transplantation Proceedings XIX*, Supp. 6: 17-22, incorporated herein by reference. Dosage forms for external application may be prepared essentially as described in EPO patent publication No. 423,714, incorporated herein by reference. The active compound is included in the pharmaceutical composition in an amount sufficient to produce the desired effect upon the disease process or condition.

For the treatment of conditions and diseases relating to immunosuppression or neuronal damage, a compound of the invention may be administered orally, topically,

parenterally, by inhalation spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvant, and vehicles. The term parenteral, as used herein, includes subcutaneous injections, and intravenous, intramuscular, and intrasternal injection or infusion techniques.

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Dosage levels of the compounds of the present invention are of the order from about 0.01 mg to about 50 mg per kilogram of body weight per day, preferably from about 0.1 mg to about 10 mg per kilogram of body weight per day. The dosage levels are useful in the treatment of the above-indicated conditions (from about 0.7 mg to about 3.5 mg per patient per day, assuming a 70 kg patient). In addition, the compounds of the present invention may be administered on an intermittent basis. Fig., at semi-weekly, weekly, semi-monthly, or monthly intervals.

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a formulation intended for oral administration to humans may contain from 0.5 mg to 5 g of active agent compounded with an appropriate and convenient amount of carrier material, which may vary from about 5 percent to about 95 percent of the total composition. Dosage unit forms will generally contain from about 0.5 mg to about 500 mg of active ingredient. For external administration, the compounds of the invention can be formulated within the range of, for example, 0.00001% to 60% by weight, preferably from 0.001% to 10% by weight, and most preferably from about 0.005% to 0.8% by weight. The compounds and compositions of the invention are useful in treating disease conditions using doses and administration schedules as described for tacrolimus in U.S. Patent Nos. 5,542,436; 5,365,948; 5,348,966; and 5,196,437, incorporated herein by reference. The compounds of the invention can be used as single therapeutic agents or in combination with other therapeutic agents. Drugs that can be usefully combined with compounds of the invention include one or more immunosuppressant agents such as rapamycin, cyclosporin A, FK-506, or one or more neurotrophic agents.

It will be understood, however, that the specific dosage level for any particular patient will depend on a variety of factors. These factors include the activity of the specific compound employed; the age, body weight, general health, sex, and diet of the subject; the time and route of administration and the rate of excretion of the drug; whether a drug combination is employed in the treatment; and the severity of the particular disease or condition for which therapy is sought.

A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the present invention and shall not be construed as being a limitation on the scope of the invention or claims.

5 Example 1

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Replacement of Methoxyl with Hydrogen or Methyl at C-13 of FK-520

The C-13 methoxyl group is introduced into FK-520 via an AT domain in extender module 8 of the PKS that is specific for hydroxymalonyl and by methylation of the hydroxyl group by an S-adenosyl methionine (SAM) dependent methyltransferase. Metabolism of FK-506 and FK-520 primarily involves oxidation at the C-13 position into an inactive derivative that is further degraded by host P450 and other enzymes. The present invention provides compounds related in structure to FK-506 and FK-520 that do not contain the C-13 methoxy group and exhibit greater stability and a longer half-life *in vivo*. These compounds are useful medicaments due to their immunosuppressive and neurotrophic activities, and the invention provides the compounds in purified form and as pharmaceutical compositions.

The present invention also provides the novel PKS enzymes that produce these novel compounds as well as the expression vectors and host cells that produce the novel PKS enzymes. The novel PKS enzymes include, among others, those that contain an AT domain specific for either malonyl CoA or methylmalonyl CoA in module 8 of the FK-506 and FK-520 PKS. This example describes the construction of recombinant DNA compounds that encode the novel FK-520 PKS enzymes and the transformation of host cells with those recombinant DNA compounds to produce the novel PKS enzymes and the polyketides produced thereby.

To construct an expression cassette for performing module 8 AT domain replacements in the FK-520 PKS, a 4.6 kb SphI fragment from the FK-520 gene cluster was cloned into plasmid pLitmus 38 (a cioning vector available from New England Biolabs). The 4.6 kb SphI fragment, which encodes the ACP domain of module 7 followed by module 8 through the KR domain, was isolated from an agarose gel after digesting the cosmid pKOS65-C31 with Sph I. The clone having the insert oriented so the single SacI site was nearest to the SpeI end of the polylinker was identified and designated as plasmid pKOS60-21-67. To generate appropriate cloning sites, two linkers were ligated sequentially as follows. First, a linker was ligated between the SpeI and SacI sites to introduce a BgIII site at the 5' end of the cassette, to eliminate interfering polylinker sites, and to reduce the total insert size to 4.5 kb (the limit of the phage

KC515). The ligation reactions contained 5 picomolar unphosphorylated linker DNA and 0.1 picomolar vector DNA, i.e., a 50-fold molar excess of linker to vector. The linker had the following sequence:

5'-CTAGTGGGCAGATCTGGCAGCT-3' 3'-ACCCGTCTAGACCG-5'

The resulting plasmid was designated pKOS60-27-1.

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Next, a linker of the following sequence was ligated between the unique *Sphī* and *Afl*II sites of plasmid pKOS60-27-1 to introduce an *Nsi*I site at the 3' end of the module 8 cassette. The linker employed was:

5'-GGGATGCATGGC-3'
3'-GTACCCCTACGTACCGAATT-5'

The resulting plasmid was designated pKOS60-29-55.

To allow in-frame insertions of alternative AT domains, sites were engineered at the 5' end (Avr II or Nhe I) and 3' end (Xho I) of the AT domain using the polymerase chain reaction (PCR) as follows. Plasmid pKOS60-29-55 was used as a template for the PCR and sequence 5' to the AT domain was amplified with the primers SpeBgl-fwd and either Avr-rey or Nhe-rey:

SpeBgl-fwd 5'-CGACTCACTAGTGGGCAGATCTGG-3'

Avr-rev 5'-CACGCCTAGGCCGGTCGGTCTCGGGCCAC-3'

Nhe-rev 5'-GCGGCTAGCTGCTCGCCCATCGCGGGATGC-3'

The PCR included, in a 50 μl reaction, 5 μl of 10x Pfu polymerase buffer (Stratagene), 5 μl 10x z-dNTP mixture (2 mM dATP, 2 mM dCTP, 2 mM dTTP, 1 mM dGTP, 1 mM 7-deaza-GTP), 5 μl DMSO, 2 μl of each primer (10 μM), 1 μl of template DNA (0.1 μg/μl), and 1 μl of cloned Pfu polymerase (Stratagene). The PCR conditions were 95°C for 2 min., 25 cycles at 95°C for 30 sec., 60°C for 30 sec., and 72°C for 4 min., followed by 4 min. at 72°C and a hold at 0°C. The amplified DNA products and the Litmus vectors were cut with the appropriate restriction enzymes (Bg/II and AvrII or Spel and Nhel), and cloned into either pLitmus 28 or pLitmus38 (New England Bio¹abs), respectively, to generate the constructs designated pKOS60-37-4 and pKOS60-37-2, respectively.

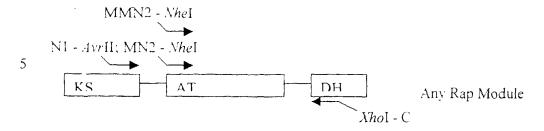
Plasmid pKOS60-29-55 was again used as a template for PCR to amplify sequence 3' to the AT domain using the primers BsrXho-fwd and NsiAfl-rev:

BsrXho-fwd 5'-GATGTACAGCTCGAGTCGGCACGCCCGGCCGCATC-3'
NsiAfl-rev 5'-CGACTCACTTAAGCCATGCATCC-3'

PCR conditions were as described above. The PCR fragment was cut with BsrGI and AfIII, gel isolated, and ligated into pKOS60-37-4 cut with Asp718 and AfIII and

inserted into pKOS60-37-2 cut with *BsrGI* and *AfIII*, to give the plasmids pKOS60-39-1 and pKOS60-39-13, respectively. These two plasmids can be digested with *AvrII* and *XhoI* or *NheI* and *XhoI*, respectively, to insert heterologous AT domains specific for malonyl, methylmalonyl, ethylmalonyl, or other extender units.

- Malonyl and methylmalonyl-specific AT domains were cloned from the rapamycin cluster using PCR amplification with a pair of primers that introduce an *Avr*II or *Nhe*l site at the 5' end and an *Xho*I site at the 3' end. The PCR conditions were as given above and the primer sequences were as follows:
- 10 RATN1 5'-ATCCTAGGGGGGCRGGYGTGTCGTCCTTCGG-3'
 (3' end of Rap KS sequence and universal for malonyl and methylmalonyl CoA),
 RATMN2 5'-ATGCTAGCCGCCGCGTTCCCCGTCTTCGCGCG-3'
 (Rap AT shorter version 5'- sequence and specific for malonyl CoA),
 RATMMN2 5'-ATGCTAGCGGATTCGTCGGTGGTGTTCGCCGA-3'
- (Rap AT shorter version 5'- sequence and specific for methylmalonyl CoA), and RATC 5'-ATCTCGAGCCAGTASCGCTGGTGYTGGAAGG-3' (Rap DH 5'- sequence and universal for malonyl and methylmalonyl CoA).



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Because of the high sequence similarity in each module of the rapamycin cluster, each primer was expected to prime any of the AT domains. PCR products representing ATs specific for malonyl or methylmalonyl extenders were identified by sequencing individual cloned PCR products. Sequencing also confirmed that the chosen clones contained no cloning artifacts. Examples of hybrid modules with the rapamycin AT12 and AT13 domains are shown in a separate figure.

The AvrII-XhoI restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 12 of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below. The AT of rap module 12 is specific for incorporation of malonyl units.

20 AGATCTGGCAGCTCGCCGAAGCGCTGCTGACGCTCGTCCGGGAGAGCACC 50 WQLA E A L L T L V GCCGCCGTGCTCGGCCACGTGGGTGGCGAGGACATCCCCGCGACGGCGGC 100 A A V L G H V G G E D I P A T A A GTTCAAGGACCTCGGCATCGACTCGCTCACCGCGGTCCAGCTGCGCAACG 150 25 F K D L G I D S L T A V Q L R N CCCTCACCGAGGGGACCGGTGTGCGGGTGAACGCCACGGGGGTCTTCGAC 200 ALTEATGVRLNATAVFD TTCCCGACCCCGCACGTGCTCGCCGGGGAAGCTCGGCGACGAACTGACCGG 250 F P T P H V L A G K L G D E L T G 30 CACCCGCGCGCCCGTCGTGCCCCGGACCGCGGCCACGGCCGGTGCGCACG 300 TRAPVVPRT A A T A G A H ACGAGCCGCTGGCGATCGTGGGAATGGCCTGCCGGCTGCCCGGCGGGGTC 350 EPLAIV G M A C R L P G G V GCGTCACCCGAGGAGCTGTGGCACCTCGTGGCATCCGGCACCGACGCCAT 400 35 A S P E E L W H L V A S G T D A I TEFPTDRGWDVDAIYD CGGACCCCGACGCGATCGGCAAGACCTTCGTCCGGCACGGTGGCTTCCTC 500 PDPDAIGKTFVRHGGFL 40 ACCGGCGCGACAGGCTTCGACGCGCGCGTTCTTCGGCATCAGCCCGCGCGA. 550 TGATGFDAAFFGISPRE GGCCCTCGCGATGGACCCGCAGCAGCGGGTGCTCCTGGAGACGTCGTGGG 600 A L A M D P Q Q R V L L E T S W AGGCGTTCGAAAGCGCCGGCATCACCCCGGACTCGACCCGCGGCAGCGAC 650 45 EAFESAGITPDSTRGSD ACCGGCGTGTTCGTCGGCGCCTTCTCCTACGGTTACGGCACCGGTGCGGA 700 TGVFVGAFSYGYGTGAD CACCGACGGCTTCGGCGCGCCGGCTCGCAGACCAGTGTGCTCTCCGGCC 750 TDGFGATGSOT SVLSG 50 GGCTGTCGTACTTCTACGGTCTGGAGGGTCCGGCGGTCACGGTCGACACG 800 RLSYFYGLEGPAVTVDT GCGTGTTCGTCGTCGCTGGTGGCGCTGCACCAGGCCGGGCAGTCGCTGCG 850 A C S S S L V A L H Q A G Q S L R

	S G E C S L A L V G G V T V M A	900
		950
	S F G G F V E F S R Q R G L A P D	, • •
5	GSCCGGGGAAGGCGTTTCGGCCGCGGGGGGCACGGCACGAGCTTCSCCGA	1000
	G R A K A F G A G A D G T S F A E	
	GGGTGCCGGTGTGCTGATCGTCGAGAGGCTCTCCGACGCCGAACGCAACG	1050
	G A G V L I V E R L S D A E R N	
	GTCACACCGTCCTGGCGGTCGTCCGTGGTTCGGCGGTCAACCAGGATGGT	1100
10	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	
	GOOTCCAACGGGCTGTCGGCGCCGAACGGGCCGTCGCAGGAGCGGGTCAT	1150
	A S N G L S A P N G P S Q E R V I	
	CCGGCAGGCCTGGCCAACGCCGGGCTCACCCCGGCGGACGTGGACGCCG	1200
, .	RQALANAGLTPADVDA	
15	TOGAGGCCACGGCACCGGCACCAGGCTGGGCGACCCCATCGAGGCACAG	1250
	V E A H G T G T R L G D P I E A Q	- 200
		1300
	A V L A T Y G Q E R A T P L L L G	1250
20		1350
20	S L K S N I G H A Q A A S G V A GCATCATCAAGATGCTGCAGGCCCTCCGGCACGGGGAGCTGCCGCCGACG	1400
	G I I K M V Q A L R H G E L P P T	1400
	CTGCACGCCGACGACGCCGCACGTCGACTGGACGGCCGGC	1450
	L H A D E P S P H V D W T A G A V	1.50
25		1500
	E L L T S A R P W P E T D R P R	
	GGGCAGGCGTGTCGTCCTTCGGGATCAGTGGCACCAACGCCCACGTCATC	1550
	R A G V S S F G I S G T N A H V I	
	CTGGAAAGCGCACCCCCACTCAGCCTGCGGACAACGCGGTGATCGAGCG	1600
30	LESAPPTQPADNAVIER	
	GGCACCGGAGTGGGTGCCGTTGGTGATTTCGGCCAGGACCCAGTCGGCTT	1650
	APEWVPLVISARTQSA	
	TGACTGAGCACGAGGGCCGGTTGCGTCGTATCTGGCGGCGTCGCCCGGG	1700
35	L T E H E G R L R A Y L A A S P G	1750
33	GTGGATATGCGGGCTGTGGCATCGACGCTGGCGATGACACGGTCGGT	1750
		1800
	E H R A V L L G D D T V T G T A	1500
	TGTCTGACCCTCGGGCGGTGTTCGTCTTCCCGGGACAGGGGTCGCAGCGT	1850
40	V S D P R A V F V F P G Q G S Q R	-000
	GCTGGCATGGGTGAGGAACTGGCCGCCGCGTTCCCCGTCTTCGCGCGGAT	1900
	A G M G E E L A A A F P V F A R I	
	CCATCAGCAGGTGTGGGACCTGCTCGATGTGCCCGATCTGGAGGTGAACG	1950
	HQQVWDLLDVPDLEVN	
45	AGACCGGTTACGCCCAGCCGGCCCTGTTCGCAATGCAGGTGGCTCTGTTC	2000
	E T G Y A Q P A L F A M Q V A L F	
	GGGCTGCTGGAATCGTGGGGTGTACGACCGGACGCGGTGATCGGCCATTC	2050
	G L L E S W G V R P D A V I G H S	0100
50	GGTGGGTGAGCTTGCGGCTATGTGCCGGGGTGTGGTCGTTGGAGG	2100
50	V G E L A A A Y V S G V W S L E	2150
	ATGCCTGCACTTTGGTGTCGGCGCGGGCTCGTCTGATGCAGGCTCTGCCC D A C T L V S A R A R L M Q A L P	2130
	GCGGGTGGGGTGATGGTCGCTGTCCCGGTCTCGGAGGATGAGGCCCGGGC	2200
	A G G V M V A V P V S E D E A R A	2200
55	CGTGCTGGGTGAGGGTGTGGAGATCGCCGCGGTCAACGGCCCGTCGTCGG	2250
	V L G E G V E I A A V N G P S S	
	TGGTTCTCTCCGGTGATGAGGCCGCCGTGCTGCAGGCCGCGGAGGGGCTG	2300
	V V L S G D E A A V L Q A A E G L	
	GGGAAGTGGACGCGCTGGCGACCAGCCACGCGTTCCATTCCGCCCGTAT	2350
60	G K W T R L A T S H A F H S A R M	
	GGAACCCATGCTGGAGGAGTTCCGGGCGGTCGCCGAAGGCCTGACCTACC	2400
	EPMLEEFRAVAEGLTY	
	GGACGCCGCAGGTCTCCATGGCCGTTGGTGATCAGGTGACCACCGCTGAG	2450
	RTPOVSMAVGDOVTTAE	

	TACTGGGTGCGGCAGGTCCGGGACACGCTCCGGTTCGGCGAGCAGGTGGC	2500
	Y,WVRQVRDTVRFGEQVA	
	CTOGTACGAGGACGCCGTGTTCGTCGAGCTGGGTGCCGACCGGTCACTGG	2550
_	S Y E D A V F V E L G A D R S L	0.600
5	COOGCOTGGTCGACGGTGTCGCGATGCTGCACGGGACCACGAAAATCCAG	2600
	A R L V D G V A M L H G D H E I Q	2000
	GCCGCGATCGGCCCCTGGCCCACCTGTATGTCAACGGCGTCACGGTCGA A A I G A L A H L Y V N G V T V D	2650
	A A I G A L A H L Y V N G V T V D CTGGCCCGCGCTCCTGGGCGATGCTCCGGCAACACGGGTGCTGGACCTTC	2700
10	W P A L L G D A P A T R V L D L	2700
10	CGACATACGCCTTCCAGCACCAGCGCTACTGGCTCGAGTCGGCACGCCG	2750
	P T Y A F Q H Q R Y W L E S A R P	2750
		2800
	A A S D A G H P V L G S G I A L A	2000
15	OGGGTCGCCAGGGTGTTCAGGGGTTCCGTGCGACCGGTGCGGACC	2850
-	G J . R V F T G S V P T G N D	
	GCGCCGTGTTCGTCGCCGAGCTGGCCGCCGCGGGGGGGGG	2900
	RAVFVAELALAAADAVD	
	TGCGCCACGGTCGAGCGGCTCGACATCGCCTCCGTGCCCGGCCGG	2950
20	CATVERLDIASVPGRPG	
	CCATGGCCGGACGACCGTACAGACCTGGGTCGACGAGCCGGCGGACGACG	3000
	H G R T T V Q T W V D E P A D D	
	GCCGGCGCCGGTTCACCGTGCACACCCGCACCGGCGACGCCCCGTGGACG	3050
	G R R F T V H T R T G D A P W T	
25	CTGCACGCCGAGGGGTGCTGCGCCCCCATGGCACGGCCCTGCCCGATGC	3100
	L H A E G V L R P H G T A L P D A	
	GGCCGACGCCGAGTGGCCCCACCGGGCGCGGTGCCCGCGGACGGCTGC	3150
	A D A E W P P P G A V P A D G L	2000
20	CGGGTGTGTGGCGCGGGGGGGACCAGGTCTTCGCCGAGGCCGAGGTGGAC	3200
30	P G V W R R G D Q V F A E A E V D	3250
	GGACCGGACGGTTTCGTGGTGCACCCCGACCTGCTCGACGCGGTCTTCTC G P D G F V V H P D L L D A V F S	3230
	G P D G F V V H P D L L D A V F S CGCGGTCGGCGACGGAGCCGCCAGCCGGCCGGATGGCGGACCTGACGG	3300
	A V G D G S R Q P A G W R D L T	3300
35	TGCACGCGTCGGACGCCACCGTACTGCGCGCCTGCCTCACCCGGCGCACC	3350
	V H A S D A T V L R A C L T R R T	
	GACGGAGCCATGGGATTCGCCGCCTTCGACGGCGCCGGCCTGCCGGTACT	3400
	D G A M G F A A F D G A G L P V L	
	CACCGCGGAGGCGTGACGCTGCGGGAGGTGGCGTCACCGTCCGGCTCCG	3450
40	TAEAVTLREVASPSGS	
	AGGAGTCGGACGGCCTGCACCGGTTGGAGTGGCTCGCGGTCGCCGAGGCG	3500
	E E S D G L H R L E W L A V A E A	
	GTCTACGACGGTGACCTGCCCGAGGGACATGTCCTGATCACCGCCGCCCA	3550
. ~	V Y D G D L P E G H V L I T A A H	
45	CCCCGACGACCCGGGGACATACCCACCGGGGCCCACACCCGGGGCCACCC	3600
	P D D P E D I P T R A H T R A T	2650
	GCGTCCTGACCGCCTCCACCACCACCACCACCACCACCACCACCACCACCAC	3650
	R V L T A L Q H H L T T 1 D H T L	3700
50	ATCGTCCACACCACCACCGACCCGCCGCGCCACCGTCACCGGCCTCAC I V H T T T D P A G A T V T G L T	3700
50	CCGCACCGCCCAGAACGAACACCCCCACCGCATCGCCTCATCGAAACCG	3750
	R T A Q N E H P H R I R L I E T	5,50
	ACCACCCCACACCCCCTCCCCCTGGCCCAACTCGCCACCCTCGACCAC	3800
	D H P H T P L P L A Q L A T L D H	
55		3850
	PHLRLTHHTLHHPHLTP	
	CCTCCACACCACCACCCACCACCACCACCCCCCTCAACCCCGAACACG	3900
	L H T T P P T T P L N P E H	
	CCATCATCATCACCGGCGGCTCCGGCACCCTCGCCGGCATCCTCGCCCGC	3950
60	A I I I T G G S G T L A G I L A R	
	CACCTGAACCACCCCACACCTACCTCCTCTCCCGCACCCCCCCGA	4000
	H L N H P H T Y L L S R T P P P D	
	CGCCACCCCGGCACCCACCTCCCCTGCGACGTCGGCGACCCCCACCAAC	4050

TCGCCACCACCCTCACCCACATCCCCCAACCCCTCACCGCCATCTTCCAC 4100 LATTLTHIPQPLTAIFH ACCGCCGCCACCCTCGACGGCATCCTCCACGCCCTCACCCCCGACCG 4150 T A A T L D D G I L H A L T P D R 5 CCTCACCACGTCCTCCACGCGARAGCGARAGGCGSCTGGCACCTGCACC 4200 LTTVLHPKANAAWELH ACCTCACCCAAAACCAACCCCTCACCCACTTCGTCCTCTACTCCAGCGCC 4250 H L T O N O P L T H F V L Y S S A GCCGCCGTCCTCGGCAGCCCCGGACAAGGAAACTACGCCGCCGCCCAACGC 4300 10 A A V L G S P G Q G N Y A A A N A CTTCCTCGACGCCTCGCCACCCCACCGCCACACCCTCGGCCAACCCGCCA 4350 F L D A L A T H R H T L G Q P A CCTCCATCGCCTGGGGCATGTGGCACACCACCAGCACCTCACCGGACAA 4400 T S I A W G M W H T T S T L T G Q 15 CTCGACGACGCCGACGGGGACGGCATCCGCCGCGGGGGGGTTTCCTCCCGAT 4450 LDDADRDRIRRGGFLPI CACGGACGACGAGGGCATGGGTATGCAT T D D E G

The AvrII-XhoI restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 13 (specific for methylmalonyl CoA) of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below.

AGATCTGGCAGCTCGCCGAAGCGCTGCTGACGCTCGTCCGGGAGAGCACC 50 25 Q L A E A L L T L V R E S T GCCGCCGTGCTCGGCCACGTGGGTGGCGAGGACATCCCCGCGACGGCGGC 100 A A V L G H V G G E D I P A T A A GTTCAAGGACCTCGGCATCGACTCGCTCACCGCGGTCCAGCTGCGCAACG 150 F K D L G I D S L T A V Q L R N 30 CCCTCACCGAGGCGACCGGTGTGCGGCTGAACGCCACGGCGGTCTTCGAC 200 ALTEATGVRLNATAVFD TTCCCGACCCGCACGTGCTCGCCGGGAAGCTCGGCGACGAACTGACCGG 250 F P T P H V L A G K L G D E L T G CACCGGGGGCGCGTCGTGCCCGGGACCGCGGGCCACGGCCGGTGCGCACG 300 35 TRAPVVPRTAATAGAH ACGAGCCGCTGGCGATCGTGGGAATGGCCTGCCGGCTGCCCGGCGGGGTC 350 DEPLAIVGMACRLPGGV GCGTCACCCGAGGAGCTGTGGCACCTCGTGGCATCCGGCACCGACGCCAT 400 ASPEELWHLVASGTDAI 40 CACGGAGTTCCCGACGGACCGCGGCTGGGACGTCGACGCGATCTACGACC 450 T E F P T D R G W D V D A I Y D CGGACCUCGACGCGATCGGCAAGACCTTCGTCCGGCACGGTGGCTTCCTC 500 PDPDAIGKTFVRHGGFL ACCGGCGCGACAGGCTTCGACGCGGCGTTCTTCGGCATCAGCCCGCGCGA 550 45 T G A T G F D A A F F G T S P R E GGCCCTCGCGATGGACCCGCAGCAGCGGGTGCTCCTGGAGACGTCGTGGG 600 A L A M D P Q Q R V L L E T S W AGGCGTTCGAAAGCGCCGGCATCACCCCGGACTCGACCCGCGCAGCGAC 650 E A F E S A G I T P D S T R G S D 50 ACCGGCGTGTTCGTCGGCGCCTTCTCCTACGGTTACGGCACCGGTGCGGA 700 GVFVGAFSYGYGTGAD CACCGACGGCTTCGGCGCGCCCGCCCAGACCAGTGTGCTCTCCGGCC 750 D G F G A T G S Q T S V L S G GGCTGTCGTACTTCTACGGTCTGGAGGGTCCGGCGGTCACGGTCGACACG 800 55 RLSYFYGLEGPAVTVDT GCGTGTTCGTCGTCGCTGGTGGCGCTGCACCAGGCCGGGCAGTCGCTGCG 850 A C S S S L V A L H Q A G Q S L R CTCCGGCGAATGCTCGCTCGCCCTGGTCGGCGGCGTCACGGTGATGGCGT 900 LALVGGVTVMA SGECS CTCCCGGCGGCTTCGTGGAGTTCTCCCGGCAGCGCGGCCTCGCGCCGGAC 950 60

SPGGFVEFSRQRGLAPD G R A K A F G A G A D G T S F A E GGGTGCCGGTGTGCTGATCGTCGAGAGGCTCTCCGACGCCAACGCAACG 1050 GAGVLIVERLSDAERN GTOACACCGTCCTGGCGGTCGTCCGTCGGCGCGCTCAAACCATCGT 1160 G H T V L A V V R G S A V N O D G GCCTCCHACGGGCTGTCGGCGCCGAACGGGCCGTCGCAGGAGGGGGTGAT 1150 ASNGLSAPNGPSOERVI 10 CCGGCAGGCCCTGGCCAACGCCGGGGTCACCCCGGCGGACGTGGACGCCG 1200 R Q A L A N A G L T P A D V D A TCGAGGCCCACGGCACCGGCACCAGGCTGGGCGACCCCATCGAGGCACAG 1250 V E A H G T G T R L G D P I E A Q GCGGTACTGGCCACCTACGGACAGGASCGCGCCACCCCCCTGCTGCTGGG 1330 15 AVLATYGOERATPLLLG OTOĞOTGAAGTOOAACATOGGOCACGCCAGGCCGCGTTCAGGCCTTCGCCC SLKSNIGHAQAASGVA GCATCATCAAGATGGTGCAGGCCCTCCGGCACGGGGAGCTGCCGCCGACG 1400 G I I K M V Q A L R H G E L P P T 20 LHADEPSPHVDWTAGAV ELLTSARPWPETDRPR GGGCGGGCGTGTCGTCCTTCGGAGTCAGCGGCACCAACGCCCACGTCATC 1850 25 R A G V S S F G V S G T N A H V I CTGGAGAGCGCACCCCCGCTCAGCCCGCGGAGGAGGCGCAGCCTGTTGA 1600 L E S A P P A Q P A E E A Q P V E GACGCCGGTGGTGGCCTCGGATGTGCTGCCGCTGGTGATATCGGCCAAGA 1650 T P V V A S D V L P L V I S A K 30 CCCAGCCCGCCTGACCGAACACGAAGACCGGCTGCGCGCCTACCTGGCG 1700 TOPALTEHEDRLRAYLA GCGTCGCCCGGGGCGGATATACGGGCTGTGGCATCGACGCTGGCGGTGAC 1750 A S P G A D I R A V A S T L A V T ACGSTCGGTGTTCGAGCACCGCGCCGTACTCCTTGGAGATGACACCGTCA 1800 35 RSVFEHRAVLLGDDTV CCGGCACCGCGGTGACCGACCCCAGGATCGTGTTTTGTCTTTCCCGGGCAG 1850 TGTAVTDPRIVFVFPGQ GGGTGGCAGTGGCTGGGGATGGGCAGTGCACTGCGCGATTCGTCGGTGGT 1900 G W Q W L G M G S A L R D S S V V 40 GTTCGCCGAGCGGATGGCCGAGTGTGCGGCGGCGTTGCGCGAGTTCGTGG 1950 F A E R M A E C A A A L R E F V ACTGGGATCTGTTCACGGTTCTGGATGATCCGGCGGTGGTGGACCGGGTT 2000 D W D L F T V L D D P A V V D R V GATGTGGTCCAGCCGGCTTCCTGGGCGATGATGGTTTCCCTGGCCGCGGT 2050 45 D V V Q P A S W A M M V S L A A V GTGGCAGGCGGCCGGTGTGCGGCCGGATGCGGTGATCGGCCATTCGCAGG 2100 W Q A A G V R P D A V I G H S Q GTGAGATCGCCGCAGCTTGTGTGGCGGGTGCGGTGTCACTACGCGATGCC 2150 G E I A A A C V A G A V S L R D A 50 GCCCGGATCGTGACCTTGCGCAGCCAGCCGGCCCCGGGGCCTGGCGGG 2200 ARIVTLRSQAIARGLAG CCGGGGCGCGATGGCATCCGTCGCCCTGCCCGCGCAGGATGTCGAGCTGG 2250 R G A M A S V A L P A Q D V E L TCGACGGGGCCTGGATCGCCGCCCACAACGGGCCCGCCTCCACCGTGATC 2300 55 V D G A W I A A H N G P A S T V I GCGGGCACCCGGAAGCGGTCGACCATGTCCTCACCGCTCATGAGGCACA 2350 A G T P E A V D H V L T A H E A Q AGGGGTGCGGGTGCGGCGATCACCGTCGACTATGCCTCGCACACCCCGC 2400 GVRVRRITVDYASHTP 60 ACGTCGAGCTGATCCGCG.3GAACTACTCGACATCACTAGCGACAGCAGC 2450 H V E L I R D E L L D I T S D S S TCGCAGACCCCGCTCGTGCCGTGGCTGTCGACCGTGGACGGCACCTGGGT 2500 SQTPLVPWLSTVDGTWV CGACAGCCGCTGGACGGGGAGTACTGGTACCGGAACCTGCGTGAACCGG 2550

D S P L D G E Y W Y R N L R E P TCGGTTTCCACCCGCCGTCAGCCAGTTGCAGGCCCAGGGCGACACCGTG 2600 V G F H P A V S O L Q A Q G D T V TTCGTCGAGGTCAGCGCGAGCCGGGTGTTGTTGCAGGCGATGGACGACGA 2650 F V E V S A S P V L L Q A M D D D TGTOGTCHCGGTTGCCACGCTGCGTCGTGACGACGGGGACGCCACCCGGA 2700 V V T V A T L R R S S S S A T R TGCTCACCCCCTGGCACAGGCCTATGTCCACGGCGTCACCGTCGACTGG 2750 M L T A L A Q A Y V H G V T V D W 10 CCCGCCATCCTCGGCACCACCACACCCGGGTACTGGACCTTCCGACCTA 2800 PAILGTTTTRVLDLPTY CGCCTTCCAACACCAGCGGTACTGGCTCGAGTCGGCACGCCCGGCCGCAT 2850 A F Q H Q R Y W L E S A R P A A CCGACGCGGGCCACCCCGTGCTGGGCTCCGGTATCGCCCTCGCCGGGTCG 2900 15 S D A G H P V L G S G I A L A G S CCGGGCCGGGTGTTCACGGGTTCCGTGCGACCGGTGCGGACCGCGCGGT 2950 PGRVFTGSVPTGADRAV GTTGGTGGCGAGGTGGCGGTGGCGGCGGGGGAGGC GGAGTGGGGCA 3000 F V A E L A L A A A D A V D C A 20 T V E R L D I A S V P G R P G H G RTTVQTWVDEPADDGRR CCGGTTCACCGTGCACACCCGCACCGGCGCGCGCGTGGACGCTGCACG 3150 25 R F T V H T R T G D A P W T L H CCGAGGGGGTGCTGCGCCCCATGGCACGGCCCTGCCCGATGCGGCCGAC 3200 A E G V L R P H G T A L P D A A D A E W P P P G A V P A D G L P G V 30 W R R G D Q V F A E A E V D G P ACGGTTTCGTGGTGCACCCCGACCTGCTCGACGCGGTCTTCTCCGCGGTC 3350 D G F V V H P D L L D A V F S A V GGCGACGGAAGCCGCCAGCCGGCCGGATGGCGCGACCTGACGGTGCACGC 3400 35 G D G S R Q P A G W R D L T V H A GTCGGACGCCACCGTACTGCGCGCCTGCCTCACCCGGCGCACCGACGGAG 3450 S D A T V L R A C L T R R T D G CCATGGGATTCGCCGCCTTCGACGGCGCCGGCCTGCCGGTACTCACCGCG 3500 AMGFAAFDGAGLPVLTA 40 GAGGCCGTGACGCTGCGGGAGGTGGCGTCACCGTCCGGCTCCGAGGAGTC 3550 EAVTLREVASPSGSEES GGACGGCCTGCACCGGTTGGAGTGGCTCGCGGTCGCCGAGGCGGTCTACG 3600 D G L H R L E W L A V A E A V Y ACGGTGACCTGCCCGAGGGACATGTCCTGATCACCGCCGCCCCACCCCGAC 3650 45 D G D L P E G H V L I T A A H P D GACCCCGAGGACATACCCACCCGCGCCCACACCCGCGCCACCCGCGTCCT 3700 D P E D I P T R A H T R A T R V L GACCGCCCTGCAACACCACCACCACCACCACCACCACCCTCATCGTCC 3750 TALQHHLTTTDHTLIV 50 ACACCACCACCGACCCGCCGGCGCCCACCGTCACCGGCCTCACCCGCACC 3800 $\label{eq:control_equation} \mathsf{H} \quad \mathsf{T} \quad \mathsf{T} \quad \mathsf{D} \quad \mathsf{P} \quad \mathsf{A} \quad \mathsf{G} \quad \mathsf{A} \quad \mathsf{T} \quad \mathsf{V} \quad \mathsf{T} \quad \mathsf{G} \quad \mathsf{L} \quad \mathsf{T} \quad \mathsf{R} \quad \mathsf{T}$ GCCCAGAACGAACACCCCCACCGCATCCGCCTCATCGAAACCGACCACCC 3850 AQNEHPHRIRLIETDHP CCACACCCCCTCCCCTGGCCCAACTCGCCACCCTCGACCACCCCCACC 3900 55 HTPLPLAQLATLOHPH LRLTHHTLHHPHLTPLH ACCACCACCCACCCACCACCACCCCCTCAACCCCGAACACGCCATCAT 4000 TTTPPTTTPLNPEHAII 60 ITGGSGTLAGILARHL ACCACCCCACACCTACCTCCTCTCCCGCACCCCACCCCCGACGCCACC 4100 NHPHTYLLSRTPPPDAT CCCGGCACCCACCTCCCCTGCGACGTCGGCGACCCCCACCAACTCGCCAC 4150

P G T H L P C D V G D P H O L A CACCOTCACCCACATOCCCCAACCCCTCACCGCCCATCTTCCACACCGCCG 4200 TLLTHIPQPLTAIFHTA CCACCCTCGACGACGCATCCTCCACGCCCTCACCCCGACCGCCTCACC 4250 ATLDDGILHALTPDRLT TVLHPKANAAWHLHHLT CCARARCCARCCCTCACCCACTTCGTCCTCTACTCCAGCGCCGCCGCCGCCG 4350 O N O P L T H F V L Y S S A A A 10 TOOTOGGCAGOCCCGGACAAGGAAACTACGCCGCCCAACGCCTTCCTC 4400 V L G S P G Q G N Y A A A N A F L GACGCCCTCGCCACCCACCCCCACACCCTCGGCCAACCCGCCACCTCCAT 4450 DALATHRHTLGQPATSI CGCCTGGGGCATGTGGCACACCACCAGCACCTCACCGGACAACTCGACG 4500 15 A W G M W H T T S T L T G Q L D ACCCCGACCGGGGACCGCGATCCGCCGCGCGCGCGGTTTCCTCCCGATCACGGAC 4550 DADRDRIRR SFLPITO GACGAGGO L'GGGGATGCAT D E G 20

The *Nhe*II-XhoI restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 12 (specific for malonyl CoA) of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below.

25 AGATCTGGCAGCTCGCCGAAGCGCTGCTGACGCTCGTCCGGGAGAGCACC 50 Q L A E A L L T L V R E S T GCCGCCGTGCTCGGCCACGTGGGTGGCGAGGACATCCCCGCGACGCGGC 100 A A V L G H V G G E D I P A T A A GTTCAAGGACCTCGCCATCGCTCACCGCGGTCCAGCTGCGCAACG 150 30 F K D L G I D S L T A V Q L R N CCCTCACCGAGGCGACCGGTGTGCGGCTGAACGCCACGGCGGTCTTCGAC 200 ALTEATGVRLNATAVFD TTCCCGACCCGCACGTGCTCGCCGGGGAAGETCGGCGACGAACTGACCGG 250 F P T P H V L A G K L G D E L T G 35 CACCCGCGCGCCCGTCGTGCCCCGGACCGCCGCCACGGCCGGTGCGCACG 300 TRAPVVPRTAATAGAH ACGAGCCGCTGGCGATCGTGGGAATGGCCTGCCGGCTGCCCGGCGGGGTC 350 DEPLAIVGMACRLPGGV GCGTCACCCGAGGAGCTGTGGCACCTCGTGGCATCCGGCACCGACGCCAT 400 40 ASPEELWHLVASGTDAI CACGGAGTTCCCGACGGACCGCGGCTGGGACGTCGACGCGATCTACGACC 450 TEFPTDRGWDVDAIYD CGGACCCCGACGCGATCGGCAGGACCTTCGTCCGGCACGGTGGCTTCCTC 500 PDPDAIGKTFVRHGGFL 45 ACCGGCGCGACAGGCTTCGACGCGGCGTTCTTCGGCATCAGCCCGCGCGA 550 TGATGFDAAFFGISPRE GGCCCTCGCGATGGACCCGCAGCAGCGGGTGCTCCTGGAGACGTCGTGGG 600 ALAMDPOORVLLETSW AGGCGTTCGAAAGCGCCGGCATCACCCCGGACTCGACCCGCGCAGCGAC 650 50 EAFESAGITPDSTR ACCGGCGTGTTCGTCGGCGCCTTCTCCTACGGTTACGGCACCGGTGCGGA 700 T G V F V G A F S Y G Y G T G A D CACCGACGGCTTCGGCGCGACCGGCTCGCAGACCAGTGTGCTCTCCGGCC 750 T D G F G A T G S O T S V L S G 55 GGCTGTCGTACTTCTACGGTCTGGAGGGTCCGGCGGTCACGGTCGACACG 800 RLSYFYGLEGPAVTVDT GCGTGTTCGTCGTCGCTGGTGGCGCTGCACCAGGCCGGGCAGTCGCTGCG 850 ACSSSLVALHQAGQSLR CTCCGGCGAATGCTCGCTCGCCCTGGTCGGCGGCGTCACGGTGATGGCGT 900 60 SGECSLALVGGVTVMA

CTCCCGGCGGCTTCGTGGAGTTCTCCCGGCAGCGGCGCCTCGCGCCGGAC 950 S P G G F V E F S R Q R G L A P D G R A K A F G A G A D G T S F A E GGGTGCCGGTGTGCTGATCGTCGAGAGGCTCTCCGACGCCGAACGCAACG 1050 G A G V L I V E R L S D A E R N GTCACACCGTCCTGGCGGTCGTCCGTGGTTCGGCGGTCAACCAGGATGGT 1100 G H T V L A V V R G S A V N Q D G GCCTCUAACGCGCTCTCGGCGCCGAACGGGCCGTCGCAGGAGCGGGTGAT 1150 10 ASNGLSAPNGPSQERVI CCGGCAGGCCCTGGCCAACGCCGGGCTCACCCCGGCGGACGTGGACGCCG 1200 R Q A L A N A G L T P A D V D A TCGAGGCCCACGGCACCGGCACCAGGCTGGGCGACCCCATCGAGGCACAG 1250 V E A H G T G T R L G D P I E A Q 15 GOGGTACTGGCCACCTACGGACAGGAGCGCGCCACCCCCTGCTGCTGGG 1300 A V L A T Y G Q E R A T P L L L G OTOGOTANA A A AAMOGGCACGCCAGGCCAGGCCGCCTCGGCGCCTCGCAG S 1 K s N I G H A Q A A S G v A GCATCATCAAGATGGTGCAGGCCCTCCGGCACGGGGGAGCTGCCGCCGACG 1400 20 G I I K M V Q A L R H G E L P P T L H A D E P S P H V D W T A G A V ELLTSARPWPETDRPR 25 GTGCCGCCGTCTCCTCGTTCGGGGTGAGCGGCACCAACGCCCACGTCATC 1550 R A A V S S F G V S G T N A H V I CTGGAGGCCGGACCGGTAACGGAGACGCCCGCGGGCATCGCCTTCCGGTGA 1600 L E A C P V T E T P A A S P S G D CCTTCCCCTGCTGGTGTCGGCACGCTCACCGGAAGCGCTCGACGAGCAGA 1650 30 LPLLVSARSPEALDEQ TCCGCCGACTGCGCCTACCTGGACACCCCCGGACGTCGACCGGGTG 1700 I R R L R A Y L D T T P D V D R V GCCGTGGCACAGACGCTGGCCCGGCGCACACACTTCGCCCACCGCGCCGT 1750 A V A O T L A R R T H F A H R A V 35 GCTGCTCGGTGACACCGTCATCACCACACCCCCGGGGACCGGCCCGACG 1800 LLGDTVITTPPADRPD AACTOGTOTTCGTCTACTCGGGCCAGGGCACCCAGCATCCCGGGATGGGC 1850 E L V F V Y S G Q G T Q H P A M G GAGCAGCTAGCCGCCGCGTTCCCCGTCTTCGCGCGGGATCCATCAGCAGGT 1900 40 EQLAAAFPVFARIHQQV GTGGGACCTGCTCGATGTGCCCGATCTGGAGGTGAACGAGACCGGTTACG 1950 WDLLDVPDLEVNETGY CCCAGCCGGCCCTGTTCGCAATGCAGGTGGCTCTGTTCGGGCTGCTGGAA 2000 A Q P A L F A M Q V A L F G L L E 45 S W G V R P D A V I G H S V G E L TGCGGCTGCGTATGTGTCCGGGGTGTGGTCGTTGGAGGATGCCTGCACTT 2100 A A A Y V S G V W S L E D A C T TGGTGTCGGCGCGGGCTCGTCTGATGCAGGCTUTGCCCLUGGGTGGGGTG 2150 50 L V S A R A R L M Q A L P A G G V ATGGTCGCTGTCCCGGTCTCGGAGGATGAGGCCCGGGCCGTGCTGGGTGA 2200 M V A V P V S E D E A R A V L G E GGGTGTGGAGATCGCCGCGGTCAACGGCCCGTCGTCGTGGTTCTCTCCG 2250 G V E I A A V N G P S S V V L S 55 GTGATGAGGCCGCCGTGCTGCAGGCCGCGGAGGGGCTGGGGAAGTGGACG 2300 G D E A A V L Q A A E G L G K W T CGGCTGGCGACCAGCCACGCGTTCCATTCCGCCCGTATGGAACCCATGCT 2350 R L A T S H A F H S A R M E P M L GGAGGAGTTCCGGGCGGTCGCCGAAGGCCTGACCTACCGGACGCCGCAGG 2400 60 E E F R A V A E G L T Y R T P Q TCTCCATGGCCGTTGGTGATCAGGTGACCACCGCTGAGTACTGGGTGCGG 2450 V S M A V G D O V T T A E Y W V R CAGGTCCGGGACACGGTCCGGTTCGGCGAGCAGGTGGCCTCGTACGAGGA 2500 Q V R D T V R F G E Q V A S Y E D

	CGCCGTGTTCGTCGAGCTGGGTGCCGACCGGTCACTGGCCCGCCTGGTCG	2550
	A V F V E L G A D R S L A R L V	0.500
	ACGSTGTCGCGGATGCTGCACGGCGACGACGACGAAATCCAGGCCGCGATCGGC	2600
5	D G V A M L H G D H E I Q A A I G GOODTSGCCACCTGTATGTCAACGGCGTCACGGTCGACTGCCCGCGCT	2650
)	A L A H L Y V N G V T V D W P A L	2030
	COTGGGGGATGCTCCGGCAACACGGGTGCTGGACCTTCCGACATACGCCT	2700
	L G D A P A T R V L D L P T Y A	
	TCCAGCACCAGCGCTACTGGCTCGAGTCGGCACGCCCGGCCGCATCCGAC	2750
10	F Q H Q R Y W L E S A R P A A S D	
	0000000000000100	2800
	A G H P V L G S G I A L A G S P G	2050
		2850
15	R V F T G S V P T G A D R A V F T G GCCGAGCTGGCCGCCGGCGGACGGTCGACTGCGCCACGGTC	2900
	V A E L A L A A A D A V D C A T V	
	ThrusgoToGACATOSh TonSTSUUGGUCGUCGGGGCCATGGCCGGAC	2950
	ERLDIAS: PGRPGHGRT	
•	GACCGTACAGACCTGGGTCGACGAGGGGGGGGGGGGGGG	3000
20	T V Q T W V D E P A D D G R R R	3050
	10001.000000000000000000000000	3050
		3100
	G V L R P H G T A L P D A A D A E	3.00
25	• • • • • • • • • • • • • • • • • • • •	3150
	W P P P G A V P A D G L P G V W	
	GCCGGGGGACCAGGTCTTCGCCGAGGCCGAGGTGGACGGAC	3200
	R R G D Q V F A E A E V D G P D G	2250
30	11001001000000000100000000	3250
30	F V V H P D L I D A V F S A V G D CGGAAGCCGCCAGCCGGCCGGATGGCGCACCTGACGGTGCACGGTCGG	3300
	G S R O P A G W R D L T V H A S	0000
	ACGCCACCGTACTGCGCGCCTGCCTCACCCGGCGCACCGACGGAGCCATG	3350
	DATVLRACLTRRTDGAM	
35	GGATTCGCCGCCTTCGACGCCGCCGGCCTGCCGGTACTCACCGCGGAGGC	3400
	G F A A F D G A G L P V L T A E A GGTGACGCTGCGGGAGGTGGCGTCACGTCCGGGTCCGAGGAGTCGGACG	3450
	V T L R E V A S P S G S E E S D	5450
	GCCTGCACCGGTTGGAGTGGCTCGCGGTCGCGAGGCGGTCTACGACGGT	3500
40	G L- H R L E W L A V A E A V Y D G	
	GACCTGCCCGAGGGACATGTCCTGATCACCGCCGCCCACCCCGACGACCC	3550
	D L P E G H V L I T A A H P D D P	2622
	CGAGGACATACCCACCGGGGCCACACGCGGCCACCGGGTCCTGACCG	3600
45	E D I P T R A H T R A T R V L T CCCTGCAACACCACCACCACCACCACCACCACCACCACCACCAC	3650
40	A L Q H H L T T T D H T L I V H T	3030
	ACCACCGACCCGCCGGCGCCACCGTCACCGGCCTCACCCGCACCGCCA	3700
	T T D P A G A T V T G L T R T A Q	
	GAACGAACACCCCCACCGCATCCGCCTCATCGAAACCGACCACCCCCACA	3750
50	NEHPHRIRLIET DHPH	2000
	CCCCCCTCCCCCTGGCCCAACTCGCCACCCTCGACCACCCCCCCC	3800
	T P L P L A Q L A T L D H P H L R CTCACCCACCACCACCACCACCACCACCACCACCACCACC	3850
	L T H H T L H H P H L T P L H T T	5050
55	CACCCCACCCACCACCCCCCCTCAACCCCGAACACGCCATCATCATCA	3900
	TPPTTTPLNPEHAIII	
	CCGGCGGCTCCGGCACCCTCGCCGGCATCCTCGCCCGCCACCTGAACCAC	3950
	T G G S G T L A G I L A R H L N H	4000
60	CCCCACACCTACCTCTCCCGCACCCCCACCCCCGACGCCACCCCCGG	4000
60	PHTYLLSRTPPPDATPGCACCACCACCACCACCCACCCACCCACCCACCCACCCA	4050
	T H L P C D V G D P H Q L A T T	.000
	TCACCCACATCCCCCAACCCCTCACCGCCATCTTCCACACCGCCGCCACC	4100

CTCGACGACGCATCCTCCACGCCCTCACCCCCGACCGCCTCACCACCGT 4150 LDDGILHALTPDRLTTV CCTCCACCCCAAAGCCAACGCCGCCTGGCACCTGCACCACACCCAAA 4200 LHPKANAAWHLHHLTQ ACCAACCCTCACCCACTTCGTCCTCTACTCCAGCGCCGCCGCCGTCCTC 4250 N Q P L T H F V L Y S S A A A V L GGCAGCCCGGACAAGGAAACTACGCCGCCGCCAACGCCTTCCTCGACGC 4300 G S P G Q G N Y A A A N A F L D A OCTOBECACOCACOGCOACACOCTOGGCCAACCOGCCACCTCCATCGCCT 4350 LATHRHTLGOPATSIA GGGGCATGTGGCACACCACCAGCACCTCACCGGACAACTCGACGACGCC 4400 W G N W H T T S T L T G Q L D D A GACCGGGACCGCATCCGCCGCGGGGGGTTTCCTCCCGATCACGGACGACGA 4450 D R D R I R R G G F L P I T D D E 15 GGGCATGGGGATGCAT

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The *NheII-XhoI* restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 13 (specific for methylmalonyl CoA) of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below.

AGATOTGGCAGCTCGCCGAAGCGCTGCTGACGCTCGTCCGGGAGAGCACC 50 QLAEALLTLVREST GCCGCCGTGCTCGGCCACGTGGGTGGCGACGACATCCCCGCGACGGCGGC 100 25 A A V L G H V G G E D I P A T A A GTTCAAGGACCTCGGCATCGACTCGCTCACCGCGGTCCAGCTGCGCAACG 150 F K D L G I D S L T A V Q L R N CCCTCACCGAGGCGACCGGTGTGCGGCTGAACGCCACGGCGGTCTTCGAC 200 ALTEATGVRLNATAVFD 30 TTCCCGACCCGCACGTGCTCGCCGGGAAGCTCGGCGACGAACTGACCGG 250 F P T P H V L A G K L G D E L T G CACCCGCGCGCCCGTCGTGCCCCGGACCGCGGCCACGGCCGGTGCGCACG 300 TRAPVVPRTAATAGAH ACGASCOGOTGGCGATCGTGGGGAATGGCCTGCCGGCTGCCCGGCGGGGTC 350 35 DEPLAIV G M A C R L P G G V GCGTCACCCGAGGAGCTGTGGCACCTCGTGGCATCCGGCACCGACGCCAT 400 ASPEELWHLVASGTDA CACGGAGTTCCCGACGGACGCGGCTGGGACGTCGACGCGATCTACGACC 450 TEFPTDRGWDVDAIYD 40 CGGACCCCGACGCGATCGGCAAGACCTTCGTCCGGCACGGTGGCTTCCTC 500 P D P D A I G K T F V R H G G F L ACCGGCGCGACAGGCTTCGACGCGGCGTTCTTCGGCATCAGCCCGCGCGA 550 TGATGFDAAFFGISPRE GGCCCTCGCGATGGACCCGCAGCAGCGGGTGCTCCTGGAGACGTCGTGGG 600 45 A L A M D P Q Q R V L L E T S W AGGCSTTCGAAAGCGCCGGCATCACCCCGGACTCGACCCGCGGCAGCGAC 650 EAFESAGITPDSTRGSD ACCGGGGTGTTCGTCGGCGCCTTCTCCTACGGTTACGGCACCGGTGCGGA 700 TGVFVGAFSYGYGTGAD 50 CACCGACGGCTTCGGCGGCGACCGGCTCGCAGACCAGTGTGCTCTCCGGCC 750 T D G F G A T G S Q T S V L S G GGCTGTCGTACTTCTACGGTCTGGAGGGTCCGGCGGTCACGGTCGACACG 800 RLSYFYGLEGPAVTVDT GCGTGTTCGTCGTCGCTGGTGGCCCTGCACCAGGCCGGGCAGTCGCTGCG 850 55 ACSSSLVALHQAGQSLR CTCCGGCGAATGCTCGCTCGCCCTGGTCGGCGGCGTCACGGTGATGGCGT 900 SGECSIALVGGVTVMA CTCCCGGCGGCTTCGTGGAGTTCTCCCGGCAGCGCGGCCTCGCGCCGGAC 950 SPGGFVEFSRQRGLAPD 60 GGCCGGGCGAAGGCGTTCGGCGCGGGTGCGGACGGCACGAGCTTCGCCGA 1000

	G R A K A F G A G A D G T S F A E GGGTGCCGGTGTGCTGATCGTCGAGAGGCTCTCCGACGCCGAACGCAACG	1050
5	G. A G V L I V E R L S D A E R N GTCACACCGTCCTGGCGGTCCGTGGTTCGGCGGTCAACCAGGATGGT G H T V L A V V R G S A V N Q D G	1100
-	GCCTCCAACGGGCTGTCGGCGCCGAACGGGCCGTCGCAGGAGCGGGTGAT	1150
	A S N G L S A P N G P S Q E R V I CCGGCAGGCCTGGCCAACGCCGGGCTCACCCCGGCGGACGTGGACGCCG R Q A L A N A G L T P A D V D A	1200
10	TOGAGGCCCACGGCACCGGCACCAGGCTGGGCGACCCCATCGAGGCACAG	1250
	GCGGTACTGGCCACCTACGGACAGGAGGGGCCACCCCCTGCTGCTGGGACACCACCCCCTGCTGCTGGGACACCACCCCCTGCTGCTGCTGGGACACCACCCCCTGCTGCTGGGACACCACCCCCTGCTGCTGGGACACCACCACCACCACCACCACCACCACCACCACCAC	1300
15	CTCGCTGAAGTCCAACATCGGCCACGCCCAGGCCGCGTCCGGCGTCGCCG S L K S N I G H A Q A A S G V A	1350
	GCATCATCAAGATGGTGCAGGGCCCTCCGGCACGGGGGGGG	1400
	CTGCACGCCGACGCCGCCGCCGCCGCCGCCGCCGCCGCCGCC	1450
20	CGAACTGCTGACGTCGGCCGGGCCGTGGCCCGAGACCGACC	1500
	GTGCCGCCGTCTCCTCGTTCGGGGTGAGCGGCACCAACGCCCACGTCATC R A A V S S F G V S G T N A H V I	1550
25	CTGGAGGCCGGACCGGTAACGGAGACGCCCGCGGCATCGCCTTCCGGTGA L E A G P V T E T P A A 5 P S G D	1600
	CCTTCCCCTGCTGGTCGGCACGCTCACCGGAAGCGCTCGACGAGCAGA L P L L V S A R S P E A L D E Q	1650
	TCCGCCGACTGCGCGCCTACCTGGACACCACCCCGGACGTCGACCGGGTG I R R L R A Y L D T T P D V D R V	1700
30	GCCGTGGCACAGACGCTGGCCCGGCGCACACACTTCGCCCACCGCGCCGT A V A Q T L A R R T H F A H R A V	1750
	GCTGCTCGGTGACACCGTCATCACCACACCCCCGCGGACCGGCCCGACG	1800
35		1850
55	GAGCAGCTAGCCGATTCGTCGGTGTTCGCCGAGCGGATGGCCGAGTG E Q L A D S S V V F A E R M A E C	1900
	TGCGGCGGCGTTGCGGAGTTCGTGGACTGGGATCTGTTCACGGTTCTGG A A A L R E F V D W D L F T V L	1950
40	ATGATCCGGCGGTGGTGACCGGGTTGATGTGCCAGCCCGCTTCCTGG D D P A V V D R V D V V O P A S W	2000
	GCGATGATGGTTTCCCTGGCCGCGGTGTGGCAGGCGGCCGGTGTGCGGCC A M M V S L A A V W Q A A G V R P	2050
45	GGATGCGGTGATCGCCAGGGTGAGATCGCCGCAGCTTGTGTGG D A V I G H S Q G E I A A A C V	2100
	CGGGTGCGGTGTCACTACGCGATGCCGCCCGGATCGTGACCTTGCGCAGC A G A V S L R D A A R I V T L R S	2150
	CAGGCGATCGCCCGGGGCCTGGCGGGCCGGGGCGCGATGGCATCCGTCGC	2200
50	Q A I A R G L A G R G A M A S V A CCTGCCCGCGCAGGATGTCGAGGTGGTCGACGGGGCCTGGATCGCCCC L P A Q D V E L V D G A W I A A	2250
	ACAACGGGCCCGCCTCACCGTGATCGCGGGCACCCCGGAAGCGGTCGAC H N G P A S T V I A G T P E A V D	2300
55	CATGTCCTCACCGCTCATGAGGCACAAGGGGTGCGGGTGCGGCGGATCAC	2350
33	H V L T A H E A Q G V R V R R I T CGTCGACTATGCCTCGCACACCCCGCACGTCGAGCTGATCCGCGACGAAC V D Y A S H T P H V E L I R D E	2400
	TACTCGACATCACTAGCGACAGCAGCTCGCAGACCCCGCTCGTGCCGTGG	2450
60	CTGTCGACCGTGGACGGCACCTGGGTCGACGGGGAGTA L S T V D G T W V D S P L D G E Y	2500
	CTGGTACCGGAACCTGCGTGAACCGGTCGGTTTCCACCCGGCGTCAGCC W Y R N L R E P V G F H P A V S	2550
	AGTTGCAGGCCCAGGGCGACACCGTGTTCGTCGAGGTCAGCGCCAGCCCG	2600

	Q L Q A Q G D T V F V E V S A S P GTGTTGTTGCAGGCGATGACGACGATGTCACGGTTGCCACGCTGCG V L L Q A M D D D V V T V A T L R	2650
5	TOGTGACGACGGCGACGCGGATGCTCACCGGCACAGGCCT R D D G D A T R M L T A L A O A	2700
	ATGTCCACGGCGTCACCGTCGACTGGCCCGCCATCCTCGGCACCACACACA	2750
	ACCOGGGTACTGGACCTTCCGACCTTCCAACACCAGCGGTACTG T R V L D L P T Y A F Q H Q R Y W	2800
10	GCTCGAGTCGGCACGCCGGCCGCATCCGACGCGGGCCACCCCGTGCTGG L E S A R P A A S D A G H P V L	2850
	GCTCCGGTATCGCCCTCGCCGGGTCGCCGGGCCGGGTGTTCACGGGTTCC G S G I A L A G S P G R V F T G S	
15	GTGCCGACCGGTGCGGGCGCGGGGTGTTCGCCGAGCTGGCGCTGGC V P T G A D R A V F V A E L A L A	
	CGCCGCGGACGGGTCGACTGCGCCACGGTCGACGTCGACATCGCCT A A D A V D C A T V E R L D I A	
20	CCGTGCCCGGCCGGCCGGGCCATGGCCLAACGACCTACAGACCTGGGTC S V P R P G H G R T T V Q T W V	3050
20		3150
25	G D A P W T L H A E G V L R P H GCACGGCCTGCCGATGCGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGGCCGACGGCCGACGGGCCGACGGCCGACGGGCCGACGGGCCGACGGGCCGACGGCCGACGGCCGACGGCCGACGGGCCGACGGCCGACGGGCCGACGGCGCGACGGCCGACGGGCCGACGGCCGACGGGCCGACGGCCGACGGCGCGACGGCCGACGGCCGACGGCCGACGGCCGACGGCGCGACGCGCGACGGCCGACGGCGCGACGCGCGACGCGACGCGACGCGACGCCGACGGGCCGACGGCGCGACGCGACGCGACGA	3200
2.	GTGCCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	3250
		3300
30	TGCTUGACGCGGTCTTCTCCGCGGTCGGCGACGGAAGCCGCCAGCCGGCC L L D A V F S A V G D G S R Q P A	3350
	$\begin{array}{cccccccccccccccccccccccccccccccccccc$	3400
35	CTGCCTCACCGGCGCACCGACGGAGCCATGGGATTCGCCGCCTTCGACG C L T R R T D G A M G F A A F D	3450
	GCGCCGGCCTGCCGGTACTCACCGCGGAGGCGGTGACGCTGCGGGAGGTG G A G L P V L T A E A V T L R E V GCGTCACCGTCCGGGCTCCGAGGAGTCGGACGGCCTGCACCGGTTGGAGTG	3500
40	A S P S G S E E S D G L H R L E W	3600
	L A V A E A V Y D G D L P E G H TCCTGATCACCGCCCCCCCCGACGACCCCGAGGACATACCCACCC	3650
	V L I T A A H P D D P E D I P T R GCCCACACCCGCGCCACCCGCGTCCTGACCGCCCTGCAACACCACCTCAC	3700
45	A H T R A T R V L T A L Q H H L T CACCACGACCACCACCACCACCACCACCACCACCACCACC	3750
	T T D H T L I V H T T T D P A G CCACCGTCACCGGCCTCACCGCCCCAGAACGAACACCCCCACCGC A T V T G L T R T A O N E H P H R	3800
50	ATCCGCCTCATCGAAACCGACACCCCCACACCCCCCTCCCCCTGGCCCA	3850
	ACTCGCCACCCTCGACCACCCCCACCTCCCCACCACCACCACCACCACCACCAC	3900
55	ACCACCCCACCTCACCCCCTCCACACCACCACCCACCCA	3950
	CCCCTCAACCCCGAACACGCCATCATCATCACCGGCGGCTCCGGCACCCT P L N P E H A I I I T G G S G T L	
	CGCCGGCATCCTCGCCCGCCACCTGAACCACCCCACACCTACCT	
60	CCCGCACCCCCCGACGCCACCCCGGCACCCACCTCCCCTGCGAC S R T P P P D A T P G T H L P C D	
	GTCGGCGACCCCACCAACTCGCCACCACCCTCACCCACACCCCCAACCC V G D P H Q L A T T L T H I P Q P	
	CCTCACCGCCATCTTCCACACCGCCGCCACCCTCGACGACGGCATCCTCC	4200

LTAIFHTAATLDDGID ACCOCCTCACCCCGACCGCCTCAC HALTPORLTTVLHPKAN GCCCCCTGGCACCTGACCCAAAACCAACCCTCACCCACTT 4300 A A W E L H H L T Q N Q P L T H H CGTCCTCTACTCCAGCGCCGCCGCCGTCCTCGGCAGCCCCGGGACAAGGAA 4350 V L Y S S A A A V L G S P G Q G N Y A A A N A F L D A L A T B R B 10 ADCOTOGGOCAACCOGOCACCTCCATCGCCTGGGGCATGTGGCACACCAC 4450 T 5 G Q P A T S I A W G M W H T 5 CAGCACCCTCACOGGACAACTCGACGACGCCGACGGGACCGCATCCGCC 4500 S T L T G Q L D D A D R D R I E GCGGGGGTTTCCTCCCGATCACGGACGACGAGGGCATGGGGATGCAT 15 RGGFLPITDDEG

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Phage KC515 DNA was prepared using the Educe described in Genetic Manipulation of *Streptomyces*. A Laboratory Manual, edited by D. Hopwood *et al.* A phage suspension prepared from 10 plates (100 mm) of confluent plaques of KC515 on *S. lividans* TK24 generally gave about 3 µg of phage DNA. The DNA was ligated to circularize at the cos site, subsequently digested with restriction enzymes *Bam*HI and *Pst*I, and dephosphorylated with SAP.

Each module 8 cassette described above was excised with restriction enzymes Bg/III and NsiI and ligated into the compatible BamHI and PsiI sites of KC515 phage DNA prepared as described above. The ligation mixture containing KC515 and various cassettes was transfected into protoplasts of Streptomyces lividans TK24 using the procedure described in Genetic Manipulation of Streptomyces, A Laboratory Manual edited by D. Hopwood et al. and overlaid with TK24 spores. After 16-24 hr, the plaques were restreaked on plates overlaid with TK24 spores. Single plaques were picked and resuspended in 200 µL of nutrient broth. Phage DNA was prepared by the boiling method (Hopwood et al., supra). The PCR with primers spanning the left and right boundaries of the recombinant phage was used to verify the correct phage had been isolated. In most cases, at least 80% of the plaques contained the expected insert. To confirm the presence of the resistance marker (thiostrepton), a spot test is used, as described in Lomovskaya et al. (1997), in which a plate with spots of phage is overlaid with mixture of spores of TK24 and phiC31 TK24 lysogen. After overnight incubation, the plate is overlaid with antibiotic in soft agar. A working stock is made of all phage containing desired constructs.

Streptomyces hygroscopicus ATCC 14891 (see US Patent No. 3,244,592, issued 5 Apr 1966, incorporated herein by reference) mycelia were infected with the recombinant phage by mixing the spores and phage (1 x 10⁸ of each), and incubating on R2YE agar (Genetic Manipulation of Streptomyces, A Laboratory Manual, edited by D.

Hopwood et al.) at 30°C for 10 days. Recombinant clones were selected and plated on minimal medium containing thiostrepton (50 µg/mi) to select for the thiostrepton resistance-conferring gene. Primary thiostrepton resistant clones were isolated and purified through a second round of single colony isolation, as necessary. To obtain thiostrepton-sensitive revertants that underwent a second recombination event to evict the phage genome, primary recombinants were propagated in liquid media for two to three days in the absence of thiostrepton and then spread on agar medium without thiostrepton to obtain spores. Spores were plated to obtain about 50 colonies per plate, and thiostrepton sensitive colonies were identified by replica plating onto thiostrepton containing agar medium. The "CP was used to determine which of the thiostrepton sensitive colonies reverted to the wild type (reversal of the initial integration event), and which contain the desired AT swap at module 8 in the ATCC 14891-derived cells. The PCR primers used amplified either the KS/AT junction or the AT/DH junction of the wild-type and the desired recombinant strains. Fermentation of the recombinant strains, followed by isolation of the metabolites and analysis by LCMS, and NMR is used to characterize the novel polyketide compounds.

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Example 2

Replacement of Methoxyl with Hydrogen or Methyl at C-13 of FK-506

The present invention also provides the 13-desmethoxy derivatives of FK-506 and the novel PKS enzymes that produce them. A variety of *Streptomyces* strains that produce FK-506 are known in the art, including *S. tsukubaensis* No. 9993 (FERM BP-927), described in U.S. Patent No. 5,624,852, incorporated herein by reference; *S. hygroscopicus* subsp. *yakushimaensis* No. 7238, described in U.S. patent No. 4,894,366, incorporated herein by reference; *S.* sp. MA6858 (ATCC 55098), described in U.S. Patent Nos. 5,116,756, incorporated herein by reference; and *S.* sp. MA 6548, described in Motamedi *et al.*, 1998, "The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK-506," *Eur. J. Biochem. 256*: 528-534, and Motamedi *et al.*, 1997, "Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK-506," *Eur. J. Biochem. 244*: 74-80, each of which is incorporated herein by reference.

The complete sequence of the FK-506 gene cluster from *Streptomyces* sp. MA6548 is known, and the sequences of the corresponding gene clusters from other FK-506-producing organisms is highly homologous thereto. The novel FK-506 recombinant gene clusters of the present invention differ from the naturally occurring gene clusters in

that the AT domain of module 8 of the naturally occurring PKSs is replaced by an AT domain specific for malonyl CoA or methylmalonyl CoA. These AT domain replacements are made at the DNA level, following the methodology described in Example 1.

The naturally occurring module 8 sequence for the MA6548 strain is shown below, followed by the illustrative hybrid module 8 sequences for the MA6548 strains.

5

	GCATGOGGCTGTACGAGGCGCACGGGGCACCGGAAGTCCCGTG3TG3TG 5	С
1.0	GCGGCCGCCTCGACGACGCGCGCACGTGCCGCTGCCGCGGGGGGCTGCG	100
10		150
		200
15	R S P C C P T T S A P T P P S R S TOOTSGAACAGCACCGCCACCTGCCCACCTGGCCGCCGAAGACAT S W N S T A T V L G H L G A E D I	250
	CCCGGCGACGACGATCGAAGGAACTCGGCATCGACTCGCTCACCGCGG	300
20	TCCAGCTGCGCAACGCGTGACCACGGCGAACGGCGTAACGCC	350
	ACAGCGGTCTTCGACTTTCCGACGCCGCGCGCGCGCGCGC	400
	CGACGAGCTGGCCGGTACCCGCGCGCGCCGGGCCGGGCC	450
25	CCGCGGCCGCGCACGACGAACCGCTGGCGATCGTGGGCATGGCCTGCCGT T A A A H D E P L A I V G M A C R	500
	CTGCCGGGCGGGTCGCGTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC L P G G V A S P Q E L W R L V A S	550
30	CGGCACCGACGCCATCACGGAGTTCCCCGCGGACCGCGGCTGGGACGTGG G T D A I T E F P A D R G W D V	
	ACGCGCTCTACGACCCGGACCCCGACGCGATCGGCAAGACCTTCGTCCGG D A L Y D P D P D A I G K T F V R	650
	H G G F L D G A T G F D A A F F G	700
35	I S P R E A L A M D P Q Q R V L	750
	TGGAGACGTCCTGGGAGGGGTTCGAAAGCGCGGGCATCACCCCGGACGCG L E T S W E A F E S A G I T P D A	
40	GCGCGGGGCAGCACACCGGCGTGTTCATCGGCGCGTTCTCCTACGGGTA A R G S D T G V F I G A F S Y G Y	
	CGGCACGCGTGCGGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA G T G A D T N G F G A T G S Q T GCGTGCTCTCCGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG	
45	S V L S G R L S Y F Y G L L G P S GTCACGGTCGACACCGCCTGCTCGTCGTCACTGGTCGCCCTGCACCACGGC	1000
40	V T V D T A C S S S L V A L H Q A AGGGCAGTCCTGCGCTCGGCGAATGCTCGCCTCGCCCTGGTCGGCGGTG	1050
	G Q S L R S G E C S L A L V G G TCACGGTGATGGCGCCGGGCGGATTCGTCGAGGTTCTCCCGGCAGCGC	
50	V T V M A S P G G F V E F S R Q R GGGCTCGCGCCGGACGGGCGGGCGGAGGCGTTCGGCGCGGGCGG	
	G L A P D G R A K A F G A G A D G TACGAGCTTCGCCGAGGGCGCCGGTGCCCTGGTGGTCGAGCGGCTCTCCG	
55	T S F A E G A G A L V V E R L S ACGCGGAGCGCCACGGCCACACGTCCTCGCCCTCGTACGCGGCTCCGCG	
55	D A E R H G H T V L A L V R G S A GCTAACTCCGACGGCGCGTCGAACGGTCTGTCGGCGCCGAACGGCCCCTC	
	A N S D G A S N G L S A P N G P S CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCG	

	Q E R V I H Q A L A N A K L T P CCGATGTCGACGGGGTCGACGGCACGGCACGGCACCGGCCTCGGCGAC	1400
5	A D M D A M E A H G T G T R L G D CCCATCSAGGCGTAGGCGCTSCTCGCGACGTACGGACGGGGGGAC P I E A C A L L A T Y G O D R A T	1450
	GCCCCTGCTGCTGGGCTCGCTGAAGTCGAACATCGGGCGAGGCCGAGGCCG	1500
	CGTCAGGGGTCGCCGGGATCATCARGATGGTGCAGGGCATCCGGCACGGG	1550
10	GAACTGCCGCCGACACTGCACGCGGACGACGTCGACTG E L P P T L H A D E P S P H V D W	1600
	GACGGCCGGTGGAGCTCCTGACGTCGGCCGGCCGTGGCCGGGGA T A G A V E L L T S A R P W P G	1650
15	COGGTCGCCGCGCGCGCGCGCGCGCGCGCGCGCACG T G R P R A A V S S F G V S G T	1700
	AACGCCCACATCATCCTTGAGGCAGGACCGGTCAAAACGGGACCGGTCGA N A H I I L E A G P V K T G P V E	1750
20	A G A I E A B P V E V G P V E A	1800
20	GACCGCTCCCCGCGGGGCGCCGCGTCAGCACCGGGGGGAAGACCTTCCGCTG G P L P A A P P S A P G E D L P L	1000
	CTOGTGTGGGGGGTTCCCCGGGGGGAGCACTCGACGAGCAGATCGGGGGGCCT L V S A R S F E A L D E Q I G R L GGGGGGCTATCTGGACACGGGGGGGGGGGGGGGGGGGGG	1900
25	R A Y L D T G P G V D R A A V A AGACACTGGCCGGGGTAGGCACTTCACCCACCGGGCGTACTGCTCGGG	2000
	Q T L A R R T H F T H R A V L L G GACACCGTCATCGGCGTCCCCCCGCGGGACCAGGCCGACGAACTCGTCTT	2050
30	D T V I G A P P A D Q A D E L V F CSTCTACTCCGGTCAGGGCACCCAGCATCCCGGGATGGGCGAGCAACTCG	2100
	V Y S G Q G T Q H P A M G E Q L CGGCCGCGTTCCCCGTTTCGCCGATGCCTGGCACGACGCGCTCCGACGS	2150
	A A A F P V F A D A W H D A L R R CTCGACGACCCGACCGCGCACGCCCCACACGGAGCCAGCACACGCTCTT	2200
35	L D D P D P H D P T R S Q H T L F CGCCCACCAGGGGGGGGTTCACCGCCCTCCTGAGGTCCTGGGACATCACGC	2250
	A H Q A A F T A L L R S W D I T CGCACGCCGTCATCGGCCACTCGGCGAGATCACCGCCGCGTACGCC	2300
40		2350
	A G I L S L D D A C T L I T T R A CCGCCTCATGCACACGCTTCCGCCGCCCGGCGCCATGGTCACGGTGCTGA R L M H T L P P P G A M V T V L	2400
45	CCAGCGAGGAGGAGGCCCTCAGGCGCTGCGGCCGGGCGTGGAGATCGCC T S E E E A R Q A L R P G V E I A	2450
	GCGGTCTTCGGCCCGCACTCCGTCGTGCTCTCGGGCGACCTGGACGCCGT A V F G P H S V V L S G D E D A V	2500
	GCTCGACGTCGCACAGCGGCTCGGCATCCACCACCGTCTGCCCGCGCCGC	2550
50	ACGCGGGCCACTCCGCGCACATGGAACCCGTGGCCGCCGAGCTGCTCGCC	2600
	ACCACTCGCGAGCTCCGTTACGACCGGCCCACACCGCCATCCCGAACGA T T R E L R Y D R P H T A I P N D	
55	CCCACCACCGCCGAGTACTGGGCCGAGCAGGTCCGCAACCCCGTGCTGT PTTAEYWAEQVRNPVL	
	TCCACGCCCACACCCAGCGGTACCCCGACGCGTGTTCGTCGAGATCGGC F H A H T Q R Y P D A V F V E I G	
60	CCCGGCCAGGACCTCTCACCGCTGGTCGACGGCATCGCCCTGCAGAACGG PGQDLSPLVDGIALQNG	
υυ	CACGGGGGACGAGGTGCACGCGCTGCACACGCGCTCTCA T A D E V H A L H T A L A R L F CACGGGGGCCACGCTCGACTGGTCCGGCATCCTCGGGGGTGCTTCGCG	
	T R G A T L D W S R I L G G A S R CACGACCCTGACGCCTCGTACGCGTTCCAGCGCGTCCCTACTGGAT	
	Checkbord and the control of the con	2,500

	H D P D V P S Y A F Q R R P Y W I CGAGTCGGCTCCCCGGCCACGGCCGACGCGGCCAC E S A P P A T A E S G H P V L G	3000
5	E S A P P A T A D S G H P V L G COGGAGTOGOCGTOGOCGGGTOGOCGGGTCTCACGGGTCCCGTG T G V A V A G S P G F V F T G P V	3050
	GCCGCCGCTCCGCCGCCGCGCGCCGCCGCCGCCGCCGCCG	3100
	COCCGACGCCACCGACTGCCCCACGGTCGACGTCGACGTCGGGACGCCACCGACTGCGCACCTCCGGACGACGACGTCGACAGCTCGACGTCGACGTCGACGTCGACGTCGGACGTCGACGTCGGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACGTCGACACGTCGACACACGTCGACACACAC	3150
10	TGCCCGGCGGCCGCCGCGCAGGGCCAGGGCGAGCCTGGGTCGAT V P G G S A R G R A T A O T W V D	3200
	GAACCOGCCGCGACGGGGGGGGGCGCTTCACCGTCCACACCCGGGTCGG E P A A D G R R R F T V H T R V G	3250
15	CGACGCCCGTGGACGCTGCACGCCGAGGGGGGTTCTCCGGCCCGGCCGCGCGCG	3300
	TGCCCCACCCGAAGCCGTCGACACCGCCTGGCCCCCGCGCGGTG V P Q P E A V D T A W P P P G A V	3350
	PADGLPGAWRRALQVFV	3400
20	CGAAGCCGAAGTCGACAGCCTGACGGCTTCGTGGCACACCCGACCTGC E A E V D S P D G F V A H P D L	3450
	TOGACGOGGTCTTCTCCGCGGTCGGCGACGGGAGCCGCCGACCGGA L D A V F S A V G D G S R Q F T G	3500
25	TGGCGCGACCTCGCGGTGCACGCGTGCTGCGCGCCTG W R D L A V H A S D A T V L R A C	3550
	CCTCACCGCCGCGACAGTGGTGTCGTGGAGCTGGCGCGCCTTCGACGGTG L T R R D S G V V E L A A F D G	3600
20	CCGGAATGCCGCTCACCGCGGAGTCCGTGACGCTGGGCGAGGTCGCG A G M P V L T A E S V T L G E V A	3650
30	TCGGCAGGCGATCCGACGAGTCGGACGGTCTGCTTCGGCTTGAGTGGTT S A G G S D E S D G L L R L E W L	3700
	P V A E A H Y D G A D E L P E G	3750
35	ACACCCTCATCACCGCCACACCCCGACGACCCGACGACCCACCAAC Y T L I T A T H P D D P D D P T N	3800
	PHNTPTRTHTQTTRVLT	3850
40	CGCCCTCCAACACCACCTCATCACCACCACCACACCACCTCATCGTCCACA A L Q H H L I T T N H T L I V H	3900
40	CCACCACCACCCCCAGGCGCCGCCGCACCGCACCGCAC	3950
	CAAAACGAACACCCGGGCGCATCCACCTCATCGAAACCCACCC	
45	CACCCCACTCCCCCTCACCCAACTCACCACCCACCCACC	
	GCCTCACCAACACCCTCCACACCCCCJACCTCACCCCCATCACCACC	
	CACCACACACCACCACACCCCCAACACCCCCACCCCTCAACCCCAA H H N T T T T P N T P P L N P N	4150
50	CCACGCCATCCTCATCACCGGCGGCTCCGGCACCCTCGCCGGCATCCTCG H A I L I T G G S G T L A G I L	4200
	CCCGCCACCTCAACCACCCCACACCTACCTCCTCTCCCGCACACCACACACA	4250
55	CCCCCCACACACCCGGCACCCACATCCCCTGCGACCTCACCGACCCCAC P P T T P G T H I P C D L T D P T	
	CCAAATCACCCAAGCCCTCACCCACATACCACAAGCCCTCACGGCATCT Q I T Q A L T H I P Q P L T G I	4350
	F H T A A T L D D A T L T N L T P	4400
60	CAACACCTCACCACCCTCCAACCCAAAGCCGACGCCGCCTGGCACCT Q H L T T T L Q P K A D A A W H L	4450
	CCACCACCACACCAAAACCAACCCCTCACCCACTTCGTCCTCTACTCCA H H H T Q N Q P L T H F V L Y S	4500
	GCGCCGCCGCCACCCTCGGCAGCCCGGCCAAGCCAACTACGCCGCCGCC	4550

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The AvrII-XhoI hybrid FK-506 PKS module 8 containing the AT domain of module 12 of rapamycin is shown below.

GCATGCGGCTGTACGAGGCGGCACGGCACCGGAAGTCCCGTGGTGGTG 50 M R L Y E A A R R T G S P V V V 15 DOGGCCGCGCTCGACGACGCCGCGCGGACGTGCCGCCTGCTGCGCGGGCTGCG 100 a A A L D D A P D V P L . 1 L R GUGTACGACCGTCCGGCGTGCCGCGTCCGGGAACGCTCTCTUGUCCACC 18 RTTVRRAAVREFSLAD 20 RSPCCPTTSAPTPPSRS TOOTGGAACAGCACCGCCACCGTGDTCGGCCACCTGGGCCGAAGACAT 250 S W N S T A T V L G H L G A E D I CCCGGCGACGACGACGTTCAAGGAACTDGGCATCGACTCGCTCACCGCGG 300 PATTTFKELGICSLTA 25 TOCAGOTGOGCAACGOGCTGACCACGOGGACCGGGGTACGCCTCAACGCC 350 V Q L R N A L T T A T G V R L N A TAVFDFPTPRALAARLG 30 D E L A G T R A P V A A R T A A CCGCGGCCGCACGACGACCGCTGGCGATCGTGGCCATGCCTT 500 TAAAHDEPLAIVGMACR CTGCCGGGGGGGTCGCCTCGCCACAGGAGCTGTGGCGTCTCGTCGGCGTC 550 L P G G V A S P O E L W R L V A S 35 CGGCACCGACGCCATCACGGAGTTCCCCGCGGACCGCGGCTGGGACGTGG 600 G T D A I T E F P A D R G W D V ACGCGCTCTACGACCCGGACCCGGACGCGATCGGCAAGACCTTCGTCCGG 650 DALYDPDPDAIGKTFVR CACGGCGGCTTCCTCGACGGTGCGACCGGCTTCGACGCGCGTTCTTCGG 700 H G G F L D G A T G F D A A F F G 40 GATCAGCCGCGCGAGGCCCTGGCCATGGACCCGCAGCAACGGGTGCTCC 750 I S P R E A L A M D P Q Q R V L TGGAGACGTCCTGGGAGGCGTTCGAAAGCGCGGGGCATCACCCCGGACGCG 800 L E T S W E A F E S A G I T P D A GCGCGGGCAGCGACACCGGCGTGTTCATCGGCGCGTTTCTCCTACGGGTA 850 ARGSDTGVFIGAFSYGY CGGCACGGGTGCGGATACCAACGGCTTCGGCGCGAJAGGGTCGCAGACCA 900 G T G A D T N G F G A T G S Q T GCGTGCTCTCCGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950 50 S V L S G R L S Y F Y G L E G P S GTCACGGTCGACACCGCCTGCTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000 V T V D T A C S S S L V A L H Q A AGGGCAGTCCCTGCGCTCGGGCGAATGCTCGCTCGCCCTGGTCGGCGGTG 1050 G Q S L R S G E C S L A L V G G TCACGGTGATGGCGTCGCCCGGCGGATTCGTCGAGTTCTCCCGGCAGCGC 1100 55 V T V M A S P G G F V E F S R Q R GGGCTCGCGCGGACGGGCGGGCGGAAGGCGTTCGGCGCGGGCGCGGACGG 1150 G L A P D G R A K A F G A G A D G TACGAGCTTCGCCGAGGGCGCCGGTGCCCTGGTG3TCGAGCGGCTCTCCG 1200 60 T S F A E G A G A L V V E R L S ACGCGGAGCGCCACGCCACACCGTCCTCGCCCTCGTACGCGGCTCCGCG 1250 DAERHGHTVLALVRGSA

GCTAACTCCGACGGGGGGTCGAACGGTCTGTCGGGGGCGGAACGGCCCCTC 1300 A N S D G A S N G L S A P N G P S CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCCG 1350 QERVIHQALANAKLT P COGATETOSACECESTOSAGECESACESEACECESCACCEGCTCSGCGAC 1400 A D V D A V E A H G T S T R L G D CCONTRONGOCGENGGCGCCCCTGGCGACGCNCGANGAGCNCCGGGGGGAC 1450 PIEAQALLATYSQDRAT GCCCCTGCTGCTCGCTCGCTGAAGTCGAACATCGGGCAGGCCAGGCCAGGCCG 1500 10 PLLLGSLKSNIGHAQA CGTCAGGGGTCGCCGGGATCATCAAGATGGTGCAGGCCATCCGGCACGG3 1550 A S G V A G I I K M V Q A I R H G GAACTGCCGCCGACACTGCACGCGGACGACGTCGCCGCACGTCGACTG 1600 E L P P T L H A D E P S P H V D W 15 TAGAVELLTSARPWPG OCGGTCGCCCTACCCCCCCGCGCGTGTCGTCCTTCGGGGATCACTGGCACC 1700 TGRFLLAGVSSFGISG. AACGCCCADGTCATCCTGGAAAGCGDACCCCCCACTCAGCCTGCGGACAA 1750 20 NAHVILESAPPTQPADN CGCGGTGATCGAGCGGGCACCGGAGTGGGTGCCGTTGGTGATTTCGGCCA 1800 A V I E R A P E W V P L V I S A RTQSALTEHEGRLRAYL 25 GCGCCTCGCCCGCGCGTGCATATGCGGGGCTGTGGCATCGACGCTGGCGAT 1900 A A S P G V D M R A V A S T L A M GACACGGTCGGTGTTCGAGCACCGTGCCGTGCTGCTGCTGGGAGATGACACCG 1950 T R S V F E H R A V L L G D D T TCACCGGCACCGCTGTGTCTGACCCTCGGGCGGTGTTCGTCTTCCCGGGA 2000 30 V T G T A V S D P R A V F V F P G CAGGGGTCGCAGCGTGCTGGCATGGGTGAGGAACTGGCCGCCGCGTTCCC 2050 Q G S Q R A G M G E E L A A A F P CGTCTTCGCGCGGATCCATCAGCAGGTGTGGGACCTGCTCGATGTGCCCG 2100 V F A R I H Q Q V W D L L D V P 35 ATCTGGAGGTGAACGAGACCGGTTACGCCCAGCCGGCCCTGTTCGCAATG 2150 DLEVNETGYAQPALFAM CAGGTGGCTCTGTTCGGGCTGCTGGAATCGTGGGGTGTACGACCGGACGC 2200 Q V A L F G L L E S W G V R P D A GGTGATCGGCCATTCGGTGGGTGAGCTTGCGGCTGCGTATGTGTCCGGGG 2250 40 V I G H S V G E L A A A Y V S G TGTGGTCGTTGGAGGATGCCTGCACTTTGGTGTCGGCGCGGGGCTCGTCTG 2300 V W S L E D A C T L V S A R A R L ATGCAGGCTCTGCCCGCGGGTGGGGTGATGGTCGCTGTCCCGGTCTCGGA 2350 M Q A L P A G G V M V A V P V S E 45 GGATGAGGCCCGGCCGTGCTGGGTGAGGSTGTGGAGATCGCCGCGGTCA 2400 DEARAVLGEGVEIAAV ACGGCCCGTCGTCGCTGCTCTCTCCGGTGATGAGGCCGCCGTGCTGCAG 2450 N G P S S V V L S G D E A A V L Q 50 A A E G L G K W T R L A T S H A F CCATTCCGCCCGTATGGAACCCATGCTGGAGGAGTTCCGGGCGGTCGCCG 2550 H S A R M E P M L E E F R A V A AAGGCCTGACCTACCGGACGCCGCAGGTCTCCATGGCCGTTGGTGATCAG 2600 E G L T Y R T P Q V S M A V G D Q 55 GTGACCACCGCTGAGTACTGGGTGCGGCAGGTCCGGGACACGGTCCGGTT 2650 V T T A E Y W V R O V R D T V R F CGGCGAGCAGGTGGCCTCGTACGAGGACGCCGTGTTCGTCGAGCTGGGTG 2700 G E Q V A S Y E D A V F V E L G CCGACCGGTCACTGGCCCGCCTGGTCGACGGTGTCGCGATGCTGCACGGC 2750 A D R S L A R L V D G V A M L H G 60 GACCACGAAATCCAGGCCGCGATCGGCGCCCTGGCCCACCTGTATGTCAA 2800 D H E I Q A A I G A L A H L Y V N CGGCGTCACGGTCGACTGGCCCGCGCTCCTGGGCGATGCTCCGGCAACAC 2850 $\mathsf{G} \quad \mathsf{V} \quad \mathsf{T} \quad \mathsf{V} \quad \mathsf{D} \quad \mathsf{W} \quad \mathsf{P} \quad \mathsf{A} \quad \mathsf{L} \quad \mathsf{L} \quad \mathsf{G} \quad \mathsf{D} \quad \mathsf{A} \quad \mathsf{P} \quad \mathsf{A} \quad \mathsf{T}$

	GGGTGGTGGACGTTCCGACATACGCCTTCCAGCAGCAGCGCTACTGGCTC R V L D L P T Y A F Q E C R Y W L	2900
	GAGTOSCOTOCOCOSCOACOSCOGOCACTOS GOODOCTOCOCOCOCOCOCOCOCOCOCOCOCOCOCOCOCOC	2950
5	E S A P P A T A D S G H P V L G T C3GAGTCSCCGGGTCSCCGGGCGGGTGTTCACGGGTCCCGTGC G V A V A G S P G R V F T G P V	3000
	COSCOSTACOSACCACOS CARTOS COGARCO CASTOCCOCOSCO PAGARANTE I A SILALIA A	3050
10	GOOGACGCACCGACTGCGCCACGGTCGAAACAGCTCCACGTCACCTCCGT A D A T D C A T V E O L D V T S V	3100
• •	GCCCGGCGGATCCGCCGCGGCAGGGCCACCGGGGAGAGACCTGGGTCGATG	3150
	AACCCCCCCCACGCCCCCCCCCCCCACACCCCCCCCCCC	3200
15	E P A A D G R R R F T V N T R V G SACGCCCCCTGGACGCCCGAGGGGGTTCTCCGGCCCGGGCCGCCT	3250
	D A P W T L H A E G V D R P G R V GCCCUAGCCCGAAAGCCCTCGACACCCCTGGCCCCGGCCGGGCGCGGGTGC	3300
	P Q P E A V D T A W P P P G A V CUBOGGEOGGEOGGEOGGEOGGEOGGEOGGEOGGEOGGEOGG	3350
20	P A D G L P G A W R R A D Q V F V GAAGDGGAAGTGGAAGGCCTGADGGCTTGGTGGCACACCCGACCTGCT	3400
	E A E V D S P D G F V A H P D L L CGACGGGGTCTTCTCCGGGGGTCGGCGACGGGAGCGGCCAGCGGACGGGAT	3450
25	D A V F S A V G D G S R Q P T G SGCGCGACCGTGCGGGCCTGC	3500
ر س	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	
	CTCACCCGCGGGACAGTGGTGTGGAGGTGGCGGCGCCTTCGACGGTGC L T R R D S G V V E L A A F D G A	3550
30	CGGAATGCCGGTGCTCACCGCGGGGTGGGTGACGCTGGGGGGGG	3600
	CGGCAGGCGGATCCGACGAGTCGGACGGTCTGCTTCGGCTTGAGTGGTTG S A G G S D E S D G L L R L E W L	3650
	COGGTGGGGGGGGCCACTACGACGGTGCCGACGACGTGCCCGAGGGCTA P V A E A H Y D G A D E L P E G Y	3700
35	CACCCTCATCACCGCCACACCCCGACGACCCGACGACCCACCACCC T L I T A T H P D D P D D P T N	3750
	CCCACAACACACCCCACACGCACCCCACACACACACACA	3800
. 40	GCCCTCCAACACCACCTCATCACCACCACCACACCACCTCATCGTCCACAC	3850
. 40	CACCACCACCACCCCAGGCCCCCCCCCCCCCCCCCCCC	3900
	AAAACGAACACCCGGCCGCATCCACCTCATCGAAACCCACCACCCCCAC	3950
45	Q N E H P G R I H L I E T H H P H ACCCCACTCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	4000
	T P L P L T Q L T T L H Q P H L R COTCACCAGAGAGACACCCTCCACACCACCTCACCCCCATCACCCCCATCACCAC	4050
	L T N N T L H T P H L T P I T T ACCACACACCACCACCACCACCACCACCACCACCACCAC	4100
50	H H D T T T T P N T P P L A J P N CACGCCATCCTCATCACCGGCGGCTCCGGCACCCTCGCCGGCATCCTCGC	4150
	H A I L I T G G S G T L A G I L A COGCCACCTCAACCACCACCACCACCTCTCCCCGCACACCACCACCAC	
55	R H L N H P H T Y L L S R T P P CCCCCACCACACCCGGCACCCCACCTCCGGACCTCACCGACCCCACC	
33	PPTTPGTHIPCDLTDPT	
	CAAATCACCCAAGCCCTCACCCACATACCACAACCCCTCACCGGCATCTT Q I T Q A L T H I P Q P L T G I F	
60	CCACACGGCGCCACCCTCGACGACGCCACCCTCACCAACCTCACCCCCCCC	
	AACACCTCACCACCACCCTCCAACCCAAAGCCGACGCCGCCTGGCACCTCQHLTTTLQPKADAAGCCGACGCCGCCTGGCACCTC	
	CACCACCACACCCAAAACCAACCCTCACCCACTTCGTCCTCTACTCCAG H H H T Q N Q P L T H F V L Y S S	4450

The AvrII-XhoI hybrid FK-506 PKS module 8 containing the AT domain of module 13 of rapamycin is shown below.

GCATGCGGCTGTACGAGGCGGCACGGCACCGGAAGTCCCGTGGTGGTG 50 15 MRLYEAARRTGSPVVV A A A L D D A P D V P L L R G L R R T T V R R A A V R E R S L A D 20 RSPCCPTTSAPTPPSRS TOOTGGAACAGCACCGCCACCGTGCTCGGCCACCTGGGCCGAAGACAT 1200 S W N S T A T V L G H L G A E D I CCCGGCGACGACGTTCAAGGAACTCGGCATCGACTCGCTCACCGCGG 300 25 PATTTFKELGIDSLTA TOCASCTGCGCAACGCGCTGACCACGGCGACCGGCGTACGCCTCAACGCC 350 V Q L R N A L T T A T G V R L N A ACAGCGGTCTTCGACTTTCCGACGCCGCGCGCGCTCGCCGCGAGACTCGG 400 TAVFDFPTPRALAARLG 30 CGACGAGCTGGCCGGTACCCGCGCGCCCGTCGCGGCCCGGACCGCGCCA 450 DELAGTRAPVAARTAA CCGCGGCCGCACGACGACGCTGGCGATCGTGGGCATGGCCTGCCGT 500 T A A A H D E P L A I V G M A C R CTGCCGGGCGGGGTCGCGTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC 550 35 L P G G V A S P O E L W R L V A S CGGCACCGACGCCATCACGGAGTTCCCCGCGGACCGCGGCTGGGACGTGG 600 GTDAITEFPADRG W D V ACGCGCTCTACGACCCGGACCCCGACGCGATCGGCAAGACCTTCGTCCGG 650 D A L Y D P D P D A I G K T F V R CACGGCGGCTTCCTCGACGGTGCGACCGGCTTCGACGCGCGTTCTTCGG 700 40 H G G F L D G A T G F D A A F F G GATCAGCCCGCGCGAGGCCCTGGCCATGGACCCGCAGCAACGGGTGCTCC 750 ISPREALAM DPQQRVL TGGAGACGTCCTGGGAGGCGTTCGAAAGCGCGGGCATCACCCCGGACGCG 800 45 LETSWEAFESAGITPDA GCGCGGGGCAGCGACACCGGCGTGTTCATCGGCGGGTTCTCCTACGGGTA 850 ARGSDTGVFIGAFSYGY CGGCACGGGTGCUGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA 900 G T G A D T N G F G A T G S Q T 50 GCGTGCTCTCCGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950 S 7 L S G R L S Y F Y G L E G P S GTCACGGTCGACACCGCCTGCTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000 V T V D T A C S S S L V A L H Q A AGGGAGTCCCTGCGCTCGGGCGAATGCTCGCTCGCCCTGGTCGGCGGTG 1050 55 GOSLRSGECSLALVGG TCACGGTGATGGCGTCGCCCGGCGGATTCGTCGAGTTCTCCCGGCAGCGC 1100 V T V M A S P G G F V E F S R Q R G L A P D G R A K A F G A G A D G TACGAGCTTCGCCGAGGGCGCCGGTGCCCTGGTGGTCGAGCGGCTCTCCG 1200 60 T S F A E G A G A L V V E R L S ACGCGGAGCGCCACGCCCACACCGTCCTCGCCCTCGTACGCGGCTCCGCG 1250

	DAERHGHTVLALVRGSA	1200
	GCTAACTCCGACGGCGCTCGAACGGTCTGTCGGCGCCCCTC A, N s D G A s N G L 3 A P N G P S	
5	COASGAACSOSTCATCCACCAGGOCCTCSOGAACSOSAVACTCACCCCCG Q E R V I H Q A L A N A K L T P	
	COGATGTOGAOGOGGTOGAGGGGACGGGACCCGGCGTOGGGGAC A D V D A V E A H G T 3 T R L G D	1400
	A D V D A V E A H G T G T R L G D GCCATCGAGGGGGAGGGGCGAGCGAC P I E A Q A L L A T Y G Q D R A T	1450
10	GODDOTGOTGOTGOGOTGARGTCGARGATGGGGGA CGCCCAGGCCG	1500
	P L L L G S L K S N I G H A Q A CGTCAGGGGTCGCCGGGATCATCAAGATGCTGCAGGCCATCCGGCACGGGA S G V A G I I K M V Q A I R H G	1550
	GAACTGCCGCCGACACTGCACGCGGACGACCGTCGCCGCACGTCGACTG	1600
15	E L P P T L H A D E P S P H V D W GACGGCCGGTGCGTCGAGGTCGAGGTCGGGGGGGGGGGG	1650
	T A G A V E L L T S A R P W F G COGGTOGOCOTAGGGGGGGGGGGGTGTCGTCCTTUGGAGTCAGGGGGAA	1700
20	T G R P R R A G V S S F G S G T	
20	AACGCCCACGTCATCCTGGAGAGCGCACCCCCGGTCAGUUCGGGGAGGA N A H V I L E S A P P A Q P A E E	1750
	GGCGCAGCCTGTTGAGACGCCGGTGGTGCCCGCTGG A O P V E T P V V A S D V b P L	1800
25	TGATATOGGCCAAGACCCAGCCCGCCCTGACCGAACACGAAGACCGGCTG	1850
23	V I S A K T Q P A L T E H E D R L GGGGCCTACUTGGCGGGGGGGGGGGGGGATATACGGGGTGTGGCATC	1900
	R A Y L A A S P G A D I R A V A S GAGGCTGGCGGTGACACGGTCGGTGTTCGAGCACGCGCGCG	1950
20	T L A V T R S V F E H R A V L L	
30	GAGATGACACCGTCACCGGCACCGCGGGTGACCGCACCCCAGGATCGTGTTT G D D T V T G T A V T D P R I V F	2000
	GTCTTTCCCGGGCAGGGGTGGCAGTGGCAGTGGGCAGTGCACTGCG	2050
	V F P G Q G W Q W L G M G S A L R CGATTCGTCGGTGGTGTTCGCCGAGCGGATGGCCGAGTGTGCGGCGGCGT	2100
35	D S S V V F A E R M A E C A A A TGCGCGAGTTCSTGGACTGGGATCTGTTCACGGTTCTGGATGATCCGGCG	2150
	racacayarrearadycraagyre.g.renaarreareedaca	
	L R E F V D W D L F T V L D D P A	
	L R E F V D W D L F T V L D D P A GTGGTGGACCGGGTTGATGTGGTCCAGCCGGCTTCCTGGGCGATGATGGT V V D R V D V V C P A S W A M M V	2200
40	GTGGTGACCGGGTTGATGTGGTCCAGCCCCCTTCCTGGSCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGGGTGTGCAGGCGGGCGGGTGTGCGGTGA	2200
40	GTGGTGACCGGGTTGATGTGGTCCAGCCCSCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGTGGCAGGCGGGTGTGGGCGGTGA S L A A V W Q A A G V R P D A V TCGGCCATTCGCAGGGTGAGATCGCGCAGCTTGTGTGGGGGGGTG	2200 2250
40	GTGGTGGACCGGGTTGATGTGGTCCAGCCCGCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGTGGCAGGCGGGTGTGGGCCGGATGCGGTGA S L A A V W Q A A G V R P D A V	2200 2250 2300
45	GTGGTGACCGGGTTGATGTGGTCCAGCCSCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGGGTGTGCAGGCGGGCGGTGTGCGGGTGA S L A A V W Q A A G V R P D A V TCGGCCATTCGCAGGGTGAGATCGCGCAGCTTGTGTGGCGGGTGG I G H S Q G E I A A A C V A G A V TCACTACGCGATGCGCGGATCGTGACCTTGCGCAGCCAGGCGATCGC S L R D A A R I V T L R S Q A I A	2200 2250 2300 2350
	GTGGTGACCGGGTTGATGTGGTCCAGCCSCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGTGCAGGCGGCGGTGTGGGCGGGTGA S L A A V W Q A A G V R P D A V TCGGCCATTCGCAGGGTGAGATCGCCGCAGCTTGTGGGGGGGTGCGGTG I G H S Q G E I A A A C V A G A V TCACTACGCGATGCCGCCGGATCGTGACCTTGCGCAGCCAGGCGATCGC S L R D A A R I V T L R S Q A I A CCGGGGCCTGGCGGGGCGGGGGGGGGGGGATCGCCCGCCC	2200 2250 2300 2350 2400
	GTGGTGACCGGGTTGATGTGGTCCAGCCSCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGTGCAGGCGGGCGGTGTGCGGCGGATGCGGTGA S L A A V W Q A A G V R P D A V TCGGCCATTCGCAGGGTGAGATCGCCGCAGCTTGTGTGGGGGGTGGGT	2200 2250 2300 2350 2400
	GTGGTGACCGGGTTGATGTGGTCCAGCCSCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGTGCAGGCGGGCGGTGTGGGGGGGG	2200 2250 2300 2350 2400 2450
45	GTGGTGACCGGGTTGATGTGGTCCAGCCSCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGTGCAGGCGGGCGGTGTGGGGGGGG	2200 2250 2300 2350 2400 2450 2500
45	GTGGTGGACCGGGTTGATGTGGTCCAGCCCSCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGTGCAGGCGGGCGGATGCGGTGA S L A A V W Q A A G V R P D A V TCGGCCATTCGCAGGGTGAGATCGCGCAGCTTGTGTGGCGGGTGGGGTG I G H S Q G E I A A A C V A G A V TCACTACGCGATGCCGCCGGATCGTGACCTTGCGCAGCCAGGCGATCGC S L R D A A R I V T L R S Q A I A CCGGGGCCTGGCGGGGCCGGGGCGGGGCGGGCCAGGCCA	2200 2250 2300 2350 2400 2450 2500
45	GTGGTGGACCGGGTTGATGTGGTCCAGCCSCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGTGCAGGCGGGCGGATGCGGTGA S L A A V W Q A A G V R P D A V TCGGCCATTCGCAGGGTGAGATCGCGCAGCTTGTGTGGCGGGTGGGT	2200 2250 2300 2350 2400 2450 2500 2550 2600
45	GTGGTGGACCGGGTTGATGTGGTCCAGCCSCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGTGGCAGGCGGCGGTGTGGGCGGGTGA S L A A V W Q A A G V R P D A V TCGGCCATTCGCAGGGTGAGATCGCGCAGCTTGTGGGGGGGG	2200 2250 2300 2350 2400 2450 2500 2650
45	GTGGTGGACCGGGTTGATGTGGTCCASCCCSCTTCCTGGGCSATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCGCGGTGTGCAGGCGGGCGGCCGGATGCGGTGA S L A A V W Q A A G V R P D A V TCGGCCATTCGCAGGGTGAGATCGCGCCAGCTTGTGTGGGGGGGTGGGT	2200 2250 2300 2350 2400 2450 2500 2550 2600 2700
45 50 55	GTGGTGGACCGGTTGATGTGGTCCAGCCGCTTCCTGGGCGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGGCAGGCGGGCGGATGCGGTGA S L A A V W Q A A G V R P D A V TCGGCCATTCGCAGGGTGAGATCGCGCAGCTTGTGTGGCGGGTGGGT	2200 2250 2300 2350 2400 2450 2500 2550 2650 2700 2750
45 50 55	GTGGTGGACCGGGTTGATGTGGTCCAGCCGCTTTCCTGGGGGATGATGGT V V D R V D V V Q P A S W A M M V TTCCCTGGCCGCGGTGGCAGGGGGGGGGGGGGGGGGGGG	2200 2250 2300 2350 2400 2450 2500 2650 2700 2750 2800

AMDDDVVTVATLRRDD GOGACGCCACCCGGATGCTCACCGCCCTGGCACAGGCCTATGTCCACGGC 2900 G D A T R M L T A L A ! A Y V H G STCACCCTQSACTGGCCCGCCATCCTCGGCACCACCACAACCCGGGTACT 2950 VTVDWFAIDGTTTRVL GGAGCTTCCGACCTACGCCTTCCAACACCACCACTTTTGCTCGACTCGG 3000 D L P T Y A F Q H Q R Y W L E S A P P A T A D S G H P U L G T G V 10 AVAGSPGRVFTGPVPAG TGCGGACCGCGCGCGTGTTCATCGCCGAACTGGCCCTCGCCGCCGCCGACG 3150 A D R A V F I A E L A L A A A D CCACCGACIGOGCCACGGTCGAACAGCTCGACGTCACCTCCGTGCCCGGC 3200 15 A T D C A T V E Q L D V T S V P G GGATCCGCCCGCGCAGGGCCACCGCGCAGACCTGGGTCGATGAACCCGC 32E0 S 3 A R G R A T A Q T W V D E P A CGCCGACGGGGCCCCGCCCCCCAAACGCGCGACGCCC 3300 A D G L R R F T V H T R V G D A 20 PWTLHAEGVLRPJRVPO PEAVDTAWPPPGAVPAD CGGGCTGCCCGGGGCGTGGCGACGCGCGGGACCAGGTCTTCGTCGAAGCCG 3450 25 G L P G A W R R A D Q V F V E A AAGTEGACAGCCTTGACGGCTTCGTESCACACCCCGACGTGCTCGACGCG 3500 E V D S P D G F V A H P D L L D A GTCTTOTOCGCGGTCGGCGACGGGAGCCGCCAGCCGATGGCGCGA 3550 V F S A V G D G S R Q F T G W R D 30 L A V H A S D A T V L R A C L T GCCGCGACAGTGGTGTCGTGGAGCTCGCCGCCTTCGACGGTGCCGGAATG 3650 R R D S G V V E L A A F D G A G M CCGGTGCTCACCGCGGAGTCGGTGACGCTGGGCGAGGTCGCGTCGGCAGG 3700 35 P V L T A E S V T L G E V A S A G CGGATCCGACGAGTCGGACGGTCTGCTTCGGCTTGAGTGGTTGCCGGTGG 3750 G S D E S D G L L R L E W L P V CGGAGGCCCACTACGACGGTGCCGAGGAGCTGCCCGAGGGCTACACCCTC 3800 A E A H Y D G A D E L P E G Y T L 40 T A T H P D D P D D P T N P H N CACACCOACACGCACCACACACAAACOACACGCGTCCTCACCGCCCTCC 3900 TPTRTHTQTTRULTAL AACACCACCTCATCACCACCACCACCACCACCTCATCGTCCACACCACCACCACC 3950 45 Q H H L I T T N H T L I V H T T T DPPGAAVTGLTRTAQNE HPGRIHLIETHHPHTP 50 TCCCCCTCACCCAACTCACCACCCTCCACCAACCCCACCTACGCCTCACC 4100 LPLTOLTTLHOPHLRLT N N T L H T P H L T P I T T H H N 55 T T T T P N T P P L N P N H A ILITGGSGTLAGILARH CTCAACCACCCCACACCTACCTCTCTCCCGCACACCACCACCCCCCAC 4300 LNHPHTYLLSRTPPPT 60 T P G T H I P C D L T D P T Q I CCCAAGCCCTCACCCACATACCACAACCCCTCACCGGCATCTTCCACACC 4400 TQALTHIPOPLTGIFHT GCCGCCACCCTCGACGACGCCACCCTCACCAACCTCACCCCCCCAACACCT 4450

A A T L D D A T L T N L T P Q H L CACCACCACCCTCCAACCCAAAGCCGACGCCGCCTGGCACCTCCACCACC 4500 T T L Q P K A D A A W H L H B ACACCCAAAACCAACCCCTCACCCACTTCGTCCTCTACTCCAGCGCCGCC 4550 H T O N Q P L T H F V L Y S S A A GCCACCCTTGGGCAGCCCCGGGCAAGCCAACTACGCCCAACGCCTT 4600 ATLGSPSÇANYAAANAF COMEGNOGOCOMOGOCACCOMACCOMAGGACAACCOGCCACCA 4600 LDALATHRHTQSQPAC 10 DENTEGESTOGGSERTGTGGCACACCACCACCACACACTCACCAGCCAACTC 4700 TIAWGMWHTTTTLTSQL T D S D R D R I R R G G F L P I S GGACGACGAGGGCATGC 15 D D E G M

The *Nhel-Xhol* hybrid FK-506 PKS module 8 containing the AT domain of module 12 of rapanycin is shown below.

GCATGCGGCTGTACGAGGCGGCACGGCACCGGAAGTCCCGTGGTGGTG 50 20 M R L Y E A A R R T G S P V V V GOGGCCGCTCGACGACGCCCCGACGTGCCGCTGCTGCGCGGGCTGCG 100 A A A L D D A P D V P L L R G L R GCGTACGACCGTCCGGCGTGCCGCCGTCCGGGAACGGTCTCTCGCCGACC 180 R T T V R R A A V R E R S L A D 25 RSPOOPTTSAPTPPSRS TOOTGGAACAGCACCGCCACCGTGCTCGGCCACCTGGGCGCCGAAGACAT 250 S W N S T A T V L G H L G A E D I CCCGGCGACGACGTTCAAGGAACTCGGCATCGACTCGCTCACCGCGG 300 30 PATTTFKELGIDSLTA TCCAGCTGCGCAACGCGCTGACCACGGCGACCGGCGTACGCCTCAACGCC 350 V Q L P N A L T T A T G V R L N A T A V F D F P T P R A L A A R L G 35 CGACGAGCTGCCGGTACCCGCGCGCCCGTCGCGGCCCGGACCGCGCCCA 450 DELAGTRAPVAARTAA CCGCGGCGCGCACGACGAACCGCTGGCGATCGTGGGCATGGCCTGCCGT 500 TAAAHDEPLAIVGMACR CTGCCGGGCGGGTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC 550 40 L P G G V A S P Q E L W R L V A S CGGCACGGACGCCATCACGGAGTTCCCCGGGGACGGGGCTGGGACGTGG 600 G T D A I T E F P A D R G W D V ACGCGCTCTACGACCCGGACCCCGACGCGATCGGCAAGACCTTCGTCCGG 650 DALYDPDPDAIGKTFVR 45 CACGGCGGCTTCCTCGACGGTGCGACGGGCTTCGACGCGGCGTTCTTCGG 700 H G G F L D G A T G F D A A F F G GATCAGCCGCGCGAGGCCCTGGCCATGGACCCGCAGCAACGGGTGCTCC 750 I S P R E A L A M D F Q Q P V L TGGAGACGTCCTGGGAGGCCTTCGAAAGCGCGGGCATCACCCCGGACGCG 800 50 L E T S W E A F E S A G I T P D A GCGCGGGCAGCGACACCGGCGTGTTCATCGGCGCGTTCTCCTACGGGTA 850 ARGSDTGVFIGAFSYGY CGGCACGGGTGCGGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA 900 G T G A D T N G F G A T G S Q T GOSTGCTCTCCGGCCCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950 55 S V L S G R L S Y F Y G L E G P S GTCACGGTCGACACCGCCTGCTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000 V T V D T A C S S S L V A L H Q A AGGGCAGTCCCTGCGCTCGGGCGAATGCTCGCTCGCCCTGGTCGGCGGTG 1050 60 G Q S L R S G E C S L A L V G G TCACGGTGATGGCGTCGCCCGGCGGATTCGTCGAGTTCTCCCGGCAGCGC 1100 V T V M A S P G G F V E F S R Q R

	GGGCTCGCGCGGACGGCGAAGGCGTTCGGCGCGGGCGGACGG	1150
	G L A P D G R A K A F G A G A D G TACQAGCTTCGGGAGGGGGGGGGGGGGGGGGGGGGGGGGG	1200
5	T S F A E G A G A L V V E R L S ACGUSTAGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	1250
	GOTAACTOOGACGGGGGGGGGAACGGTCTGTCGUGGCGGAACGGCCCCC A N S D O A S N G L S A F N G P S	1300
10	DCAGGAAGGGGTCATCCACCAGGCCCTCGTGAAGGCGAAAGCACCCCCGG Q E R V I H Q A L A N A K L T P	1350
	COBATOTOGACGCGGTCGACGCGACGGGACCGGGGACCGGGCGACCGGGGGACACCGGGGACCGGGGACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGGCACCGGCACCGGCACCAC	1400
	CCCATCGAGGCGCAGGCGCTGCTCGCGACGTACGGACAGGACCGGGGGGAC F I E A Q A L L A T Y G Q D R A T	1450
15	GCCCCTGCTGCTGGCTGGCTGAGGTCGAACATCGGGGGCGAGGCCG P L L G S L K S N I G H A Q A	1500
	CGTCAGGGGTCGCCGGGATCATCAAGMTGCAGGCCATCCGGCACGGG	1550
20	GAACTGCCGCGACACTGCACGCGGACGACGCCGCCGCCGCCGCCGCCGCCGCCGCC	1600
	GACGGCCGGTGCCGTCGAGCTCCTGACGTCGGCCGGGCGGG	
	COGGTOGCCGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	1700
25	AACGCCCACATCATCCTTGAGGCAGGACCGGTCGAAAACGGGACCGGTCGA N A H I I L E A G P V K T G P V E	1750
		1800
30		1850
	CTCGTGTCGGCGCGTTCCCCGGAGGCACTCGACGAGCAGATCGGGCGCCT L V S A R S P E A L D E Q I G R L	1900
		1950
35	AGACACTGGCCGGGCGTACGCACTTCACCCACCGGGCCGTACTGCTCGGG	2000
	Q T L A R R T H F T H R A V L L G GACACGTCATCGGGGTCCCCCCGGGGGACGACGACGACGACGACTCGTCTT	2050
	D T V I G A P P A L Q A D E L V F	0100
40	CGTCTACTCCGGTCAGGGCACCCAGCATCCCGGGATGGGCGAGCAGCTAG V Y S G Q G T Q H P A M G E Q L	
	CCGCCGCGTCTTCGCGCGGATCCATCAGCAGGTGTGGGACCTG A A A F P V F A R I H Q Q V W D L	
	CTCGATGTGCCCGATCTGGAGGTGAACGAGACCGGTTACGCCCAGCCGGC L D V P D L E V N E T G Y A Q P A	2200
45	CCTGTTCGCAATGCAGGTGGCTCTGTTCGGGCTGCTGGAATCGTGGGGTG L F A M Q V A L F G L L E S W G	2250
	TACGACCGGACGCGGTGATCGGCCATTCGGTGAGCTTGCGGCTGCGVRPDAVIGHSVGELAAAA	2300
50	TATGTGTCCGGGCTTGTGGTCGTTGGAGGATGCCTGCACTTTGGTGTCGGC	2350
50	Y V S G V W S L E D A C T L V S A GCGGGCTCGTCTGATGCAGGCTCTGCCCGCGGGGGTGGGGGTGATGGTCGCTG	2400
	TCCCGGTCTCGGAGGATGAGGCCCGGGCCGTGCTGGGTGAGGGTGTGGAG	2450
55	V P V S E D E A R A V L G E G V E ATCGCCGCGGTCAACGGCCCGTCGTCGGTGGTTCTCTCCGGTGATGAGGC I A A V N G P S S V V L S G D E A	2500
	CGCCGTGCTGCAGGCCGCGGAGGGGCTGGGGAAGTGGACGCGGCTGGCGA	2550
60	A V L Q A A E G L G K W T R L A CCAGCCACGCGTTCCATTCCGCCCGTATGGAACCCATGCTGGAGGAGTTC	2600
60	T S H A F H S A R M E P M L E E F CGGGCGTCGCCGAAGGCCTGACCTACCGGACGCCGCAGGTCTCCATGGC	2650
	R A V A E G L T Y R T P Q V S M A CGTTGGTGATCAGGTGACCACCGCTGAGTACTGGGTGCGGCAGGTCCGGG	2700
	V G D Q V T T A E Y W V R Q V R	

	ACACGSTCCGGTTCGGCGAGCAGGTGGCCTCGTACGAGGACGCCGTGTTC D T V R F G E Q V A S Y E D A V F	2750
	GTOGASCTOSCTGCCGACCGCTCACTGGCCCCCTGGTCGACGGTGTCGCCV E L G A D R S L A R L V D G V A	2800
5	GATGCTGCACGGCGACGACGAAATCCAGGCCGGCGGATCGGCCGCCCCCCCC	2850
	ACCTGTATGTCAACGGCGTCACGGTCGACTGGCCGCGCGCTCCTGGGCGAT H L Y V N G V T V D W P A L L G D	2900
10	GCTCCGGCAACACGGGTGCTGGACCTTCCGACATACGCCTTCCAGCACCA A P A T R V L D L P T Y A F Q E Q	2950
	GOGOTACTGGTTGGAGTCGGGTCCCCCCGGGCCACGGCCGACTCGGGCCACC R Y W L E S A P P A T A D S G H	3000
	CCGTCCTCGGCAGTCGCCGTCGCCGGGTCGCCGGGCCGG	3050
15	$ \begin{array}{cccccccccccccccccccccccccccccccccccc$	3100
	A L A A A D A T D C A T T E Q L	3150
20	ACGTCACCTCCGTGCCGGGGGGATCCGCCGGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGCAGGGAGGA	3200
	ACCTGGGTCGATGAACCCGCCGCCGCCGACGGGGGGGGGG	3250
	TRVGDAPWTLHAEGVL	3300
25	GCCCCGGCCGCGTGCCCCAGCCCGGAAGCCGTCGACACCCCCCGG R P G R V P Q P E A V D T A W P P	3350
	P G A V P A D G L P G A W R R A D	3400
30	CCAGGTCTTCGTCGAAGCCGAAGTCGACAGCCCTGACGCCTTCGTGGCACQ V F V E A E V D S P D G F V A	3450
	ACCCCGACCTGCTCGACGCGGTCTTCTCCGCGGTCGGCGACGGGACGGAC	
2.5	CAGCCGACCGGATGGCGCGACCTCGCGGTGCACGCGTCGGACGCCACCGT Q P T G W R D L A V H A S D A T V	
35	L R A C L T R R D S G V V E L A	3600
	CCTTCGACGGTGCCGGAATGCCGGTGCTCACCGCGGAGTCGGTGACGCTG A F D G A G M P V L T A E S V T L	
40	GGCGAGGTCGCGTCGGCAGGCGGATCCGACGAGTCGGACGGTCTGCTTCG G E V A S A G G S D E S D G L L R	3700
	GCTTGAGTGGTTGCCGGTGGCGGAGGCCCACTACGACGGTGCCGACGAGC L E W L P V A E A H Y D G A D E	3750
A E	TGCCCGAGGGCTACACCCTCATCACCGCCACACACCCCGACGACGACCCCGAC L P E G Y T L I T A T H P D D P D	
45	GACCCCACCACCACACACACCCCACACGCACCCCACACACAAACCAC D P T N P H N T P T R T H T Q T T	
	ACGCGTCCTCACCGCCCTCCAACACCACCTCATCACCACCACCACCACCACCAC	
50	L I V H T T T D P P G A A V T G L ACCOGCACCGCACAAAACGAACACCCCGGCCGCCATCCACCTCATCGAAAC	
	T R T A Q N E H P G R I H L I E T CCACCACCCCACCACCACCACCACCACCACCACCACCAC	
55	H H P H T P L P L T Q L T T L H AACCCACCTACGCCTCACCAACAACACCCCCCCCCCCCC	
JJ	Q P H L R L T N N T L H T P H L T CCCATCACCACCACCACCACCACCACCACCACCACCACCA	
60	CCTCAACCCCAACCACGCCATCCTCATCACCGGGGGCTCCGGCACCCTCG L N P N H A I L I T G G S G T L	
	CCGGCATCCTCGCCCGCCCCCCCCCCCCCCCCCCCCCCC	
	CGCACACCACCACCACCACCACCACCACCACCACCACCAC	4200

CACCGACCCCACCCAAATCACCCAAGCCCTCACCCACATACCACAACCCC 4350 TOPTQITQALTHIPQP TOACUSSCATOTTOBRORODECOBOCACOTOSACEROECACOCOTOROC 4400 GIFETAATLODATLT ANCOTORCOCCCARGACOTORCOCCCCCCCARGGGGAAGGGGACGC 4450 R L T P Q H L T T T L D P H A D A AWHLHHHTQNQPLTHF TCCTCTACTCCAGCGCCGCCGCCACCCTCGGCAGCCCGGGCCAAGCCAAC 4550 10 V L Y S S A A A T L G S P G Q A N TACGCCGCCGCCAACGCCTTCCTCGACGCCCTCGCCACCCCACCGCCACCACAC 4600 Y A A A N A F L D A L A T H R H T Q G Q P A T T I A W G M W H T T COACACTOACCAGOCIACTOACCGACAGCGACCGCGGCGCGCATCCGCCGC 4760 15 T T L T S Q L T D S D R D R I R R GGCGGCTTCCTGCCGATCTCGGACGACGAGGGCATGC GGFLPISLDEGM

GCATGCGGCTGTACGAGGCGGCACGGCACCGGAAGTCCCGTGGTGGTG 50

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The *NheI-XhoI* hybrid FK-506 PKS module 8 containing the AT domain of module 13 of rapamycin is shown below.

M R L Y E A A R R T G S P V V V GCGGCCGCCTCGACGACGCCCCCCGGACGTGCCCCTGCTGUGCGGGCTGCG 100 25 A A A L D D A P D V P L L R G L R GOGTACGACCGTCCGGCGTGCCGCCGTCCGGGAACGCTCTCTCGCCGACC 150 R T T V R R A A V R E R S L A D RSPCCPTTSAPTPPSRS 30 TCCTGGAACAGCACCGCCACCGTGCTCGGCCACCTGGGCGCCGAAGACAT 250 SWNSTATVLGHLGAEDI CCCGGCGACGACGTTCAAGGAACTCGGCATCGACTCGCTCACCGCGG 300 PATTFKELGIDSLTA TCCAGCTGCGCAACGCGCTGACCACGGCGACCGGCGTACGCCTCAACGCC 350 35 V Q L R N A L T T A T G V R L N A TAVFDFPTPRALAARLG CGACGASCTGGCCGGTACCCGCGCCCGTCGCGGCCGGACCGCGGCCGA 450 D E L A G T R A P V A A R T A A CCGCGGCCGCGCACGACGACGCTGGCGATCGTGGGCATGGCCTGCCGT 500 40 T A A A H D E P L A I V G M A C R CTGCCGGGGGGGTCGCGTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC 550 L P G G V A S P Q E L W R L V A S CGGCACCGACGCCATCACGGAGTTCCCCGCGGACCGCCGCTGGG7 noTGG 600 45 G T D A I T E F P A D R G W D V ACGCGCTCTACGACCCGGACCCCGACGCGATCGGCAAGACCTTCGTCCGG 650 D A L Y D P D P D A I G K T F V R CACGGGGGCTTCCTCGACGGTGCGACGGGCTTCGACGCGGGGTTCTTCGG 700 H G G F L D G A T G F D A A F F G 50 GATCAGCCGCGCGAGGCCCTGGCCATGGACCCGCAGCAACGGGTGCTCC 750 I S P R E A L A M D P Q Q R V L TGGAGACGTCCTGGGAGGCGTTCGAARGCGCGCGCATCACCCCGGACGCG 800 LETSWEAFESAGITPDA GCGCGGGGCAGCGACACCGGCGTGTTCATCGGCGCGTTCTCCTACGGGTA 850 55 ARGSDTGVFIGAFSYGY CGGCACGGGTGCGGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA 900 G T G A D T N G F G A T G S Q T GOGTGETCTCCGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950 S V L S G R L S Y F Y G L E G P S 60 GTCACGGTCGACACCGCCTGCTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000 V T V D T A C S S S L V A L H Q A AGGGCAGTCCCTGCGCTCGGCGAATGCTCGCTCGCCCTGGTCGGCGGTG 1050

G Q S L R S G E C S L A L V G G TCACGGTGATGGCGTCGCCCGGCGGATTCGTCGAGTTCTCCCGGCAGCGC 1100 V.T V M A S P G G F V E F S R Q R 5 G L A P D G R A K A F S A G A D G TACCAGCTTCCCCGACGCCCCGCTCCCTCGTCCTCCGACCCCTCCCG 1200 T S F A E G A G A D V V E R L S AGGGGGAGGGCGACACGGTCCTCGCCCTCGTACGGGGGCTCCGCC 1250 DAERHGHTVLALVRGSA 10 GCTAACTCCGACGGGGGGTCGAACGGTCTGTCGGGGGCCGAACGGCCCCTC 1300 A N S D G A S N G L S A P N G P S CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCCG 1350 QERVIHQALANAKLTP COGATGTOGACGCGGTCGAGGCGCACGGCACGGGCACCGGCGCCGCCGAC 1400 15 A D V D A V E A H G T S T R L G D CCCATCGAGGCGCAGGCGCTGCTCGCGACGTACGGACAGGACCGGGCGAC 1450 PIEAQALLATYGQDRAT GOCCOTGOTGOTGGGCTC. OTGAAGTUUNDATCGGGCACGCCCAGGCCG 1500 PL LG SLK SNIGHAQA 20 CGTCAGGGGCGGCGGGATCATCAAGATGGTGCAGGCCATCCGGCACGGG 1550 ASGVAGIIKMVQAIRHG GAACTGCCGCCGACACTGCACGCGGGACGTCGCCGCACGTCGACTG 1600 E D P P T L H A D E P S P H V D W GACGGCCGGTGCGTCGAGCTCCTGACGTCGGCCCGGCCGTGGCCGGGGA 1650 25 TAGAVELLTSARPWPG CCGGTCGCCGCGCGCGCGCTGCCGTTCGTCGTTCGGCGTGAGCGGCACG 1700 T G R P R R A A V S S F G V S G T AACGCCCACATCATCCTTGAGGCAGGACCGGTCAAAACGGGACCGGTCGA 1750 NAHIILEAGPVKTGPVE 30 GGCAGGAGCGATCGAGGCAGGACCGGTCGAAGTAGGACCGGTCGAGGCTG 1800 A G A I E A G P V E V G P V E A GACCGCTCCCCGCGCGCCGCCGTCAGCACCGGGCGAAGACCTTCCGCTS 1850 G P L P A A P P S A P G E 5 L P L CTCGTGTCGGCGCGTTCCCCGGAGGCACTCGACGAGCAGATCGGGCGCCT 1900 35 LVSARSPEALDEQIGRL GCGCGCCTATCTCGACACCGGCCCGGGCGTCGACCGGGCGGCGTCGTGGCGC 1950 RAYLD T G P G V D R A A V A AGACACTGGCCCGGCGTACGCACTTCACCCACCGGGCCGTACTGCTCGGG 2000 Q T L A R R T H F T H R A V L L G 40 GACACCGTCATCGGCGCTCCCCCCCGGGACCAGGCCGACGAACTCGTCTT 2050 D T V I G A P P A D Q A D E L V F CGTCMACTCCGGTCAGGGCACCCAGCATCCCGCGATGGGCGAGCAGCTAG 2100 V Y S G Q G T Q H P A M G E Q L CCGATTCGTCGGTGTTTCGCCGAGCGGATGGCCGAGTGTGCGGCGCGCG 2150 45 A D S S V V F A E R M A E C A A A TTGCGCGAGTTCGTGGACTGGGATCTGTTCACGGTTCTGGATGATCCGGC 2200 LREFVDWDLFTVLDDPA GGTGGTGGACCGGGTTGATGTGGTCCAGCCCGCTTCCTGGGCGATGATGG 2250 V V D R V D V V Q P A S W A M M 50 TTTCCCTGGCCGCGGTGTGGCAGGCGGCCGGTGTGCGGCCGGATGCGGTG 2300 V S L A A V W Q A A G V R P D A V ATCGGCCATTCGCAGGGTGAGATCGCCGCAGCTTGTGTGGCGGGTGCGGT 2350 I G H S Q G E I A A A C V A G A V GTCACTACGCGATGCCGCCCGGATCGTGACCTTGCGCAGCCAGGCGATCG 2400 55 SLRDAARIVTLRSQAI CCCGGGGCCTGGCGGGCCGGGGCGCGATGGCATCCGTCGCCCTGCCCGCG 2450 A R G L A G R G A M A S V A L P A CAGGATGTCGAGCTGGTCGACGGGGCCTGGATCGCCGCCCACAACGGGCC 2500 Q D V E L V D G A W I A A H N G P 60 CGCCTCCACCGTGATCGCGGGCACCCCGGAAGCGGTCGACCATGTCCTCA 2550 A S T V I A G T P E A V D H V L CCGCTCATGAGGCACAAGGGGTGCGGGTGCGGCGGATCACCGTCGACTAT 2600 T A H E A Q G V R V R R I T V D Y GCCTCGCACACCCCGCACGTCGAGCTGATCCGCGACGAACTACTCGACAT 2650

	A S H T P H V E L I R D E L L D I CATTAGGACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAG	2700
5	T3SACGGCACCTSSUTCGACAGCCCCCTGGACGGGGACTACTGGTACCGG	2750
-	AMCOMOGRICAMOGRICOGTTTCCACCCCCCCCCCACCACTTGCAGGC	0081
		2850
10	ASSOCIATISSACCIACGATCTCCTCACCGCTTCCCACGCTGCGTCGTGACGAC	2900
	Q A M D D D V V T V A T L R R D D GGGGAGGGGAAGGGGAAGGGGTATGTGCAGGG	2950
1.5	001010001001001000000111001000011001100110	3000
15	V T V D W P A I L G T T T T R V TGGACCTTCCGACCTACGCCTTCCAACACCAGCGGTACTGGCTCGAGTCG L D L P T Y A F Q H Q R V W S	3050
	GONOCCCGGGCCACGGCCGACTCGGGCCACCCGGTCUIGGGUAULGAACT	3107
20		3150
	STSCGSACSSCSSGSTSTTCATCGCCGAACTGGCGCTCGCCGCCGCCGAC	3300
25		3250
		3300
	COGCOGACGGGGGGGCGCCTTCACCGTCCACACCCGGCGTCGGCGACGCC A A D G R R R F T V H T R V G D A	3350
30	COGTOGACGOTGCACGACGAGGGGGGTTCTCCGGCCCGGC	3400
		3450
35	ACGSSCTGCCCGGGGGCGTGGCGACGCGCGGGACCAGGTCTTCGTCGAAGCC	3500
	-	3550
		3600
40	ACCTCCCCGTGCACGCGTCGGACGCGTGCTGCCTCACC D L A V H A S D A T V L R A C L T	3650
	CGCCGCGACAGTGTTGTCGTGGAGGTTGCCGGAAT R R D S G V V E L A A F D G A G M	3700
45	GCCGGTGCTCACCGCGGAGTCGGTGACGCTGGGCAGGTCGCGCAG P V L T A E S V T L G E V A S A	3750
43	GCGGATCCGACGAGTCGGACGGTCTGCTTCGGCTTGAGTGGTTGCCGGTG G G S D E S D G L L R L E W L P V	3800
	GCGGAGGCCCACTACGACGGTGCCGACGAGGTGCCCGAGGGCTACACCCT A E A H Y D G A D E L P E G Y T L	3850
50	CATCACCGCCACACACCCCGACGACGCCGACGACGCCACACCCCACACCCCACACCCCACACCCCCACACCCCACA	3900
	ACACACCACACACACACACACACACACACGCGTCCTCACCGCCCTC N T P T R T H T Q T T R V L T A L	3950
55	CAACACCACCTCATCACCACCAACCACCCTCATCGTCCACACCACCAC	1000
33	CGACCCCCAGGCGCCGCGCACCGCACCGCACAAAACG D P P G A A V T G L T R T A Q N	4050
	AACACCCGGCCGCATCCACCTCATCGAAACCCACCACCACCCCACACCCCA	4100
60	CTCCCCCTCACCCAACTCACCACCCACCCACCCACCCAC	4150
	CAACAACACCCTCCACACCCCACCTCACCCCATCACCACC	4200
	ACACCACACAACCACCCCAACACCCCCACCCTCAACCACACACCCC	4250

N T T T T T P N T P P L N P N H A ATCCTCATCACCGGCGGCTCCGGCACCCTCGCCGGCATCCTCGCCGCCCA 4300 I-L I T G G S G T L A G I L A R H 5 LNHPHTYLLSPTPPP T T P G T H I P G D L T D P T I ACCORAGEDETCA COORDATACCA SARCOCCTCA COGGORTETTCCACACAC 4450 TQALTHIFQPLTGIFHT 10 DGCCGCCACCCTCGACGACGCCACCCCCAACACC 4500 A A T L D D A T L T N L T P Q H TCACCACCACCCTCCAACCCAMAGOOGACGCCGCCTGGCACCTCCACCAC 4550 LTTTLQPKADAAWHLHH CACACCCAAAACCAACCCTCACCCACTTCGTCCTCTACTCCAGCGCCGC 4600 15 HTQNQPLTHFVLYSSAA 0300A000T0300AG0000GG00AAG00AAGTA0G00GGGGGCAAGGCCT 4650 ATLGSPGQANYAAANA Toomig<mark>acgccot</mark>cgccannnacgggggaaggaaggcaaccaaccaaccacaa F L D A L A T H A H T Q G Q P A T 20 ACCATOGOCTGGGGCATGTGGCACACCACCACCACACTCACCAGCCAACT 4750 T I A W G M W H T T T T L T S Q L CACCGACAGCGACCGCACCGCATCCGCCGCGGGGGGCTTCCTGCCGATCT 4800 T D S D R D R I R R G G F L P I COGACGACGAGGGCATGC 25 SDDEGM

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Example 3

Recombinant PKS Genes for 13-desmethoxy FK-506 and FK-520

The present invention provides a variety of recombinant PKS genes in addition to those described in Examples 1 and 2 for producing 13-desmethoxy FK-506 and FK-520 compounds. This Example provides the construction protocols for recombinant FK-520 and FK-506 (from *Streptomyces* sp. MA6858 (ATCC 55098), described in U.S. Patcnt Nos. 5,116,756, incorporated herein by reference) PKS genes in which the module 8 AT coding sequences have been replaced by either the *rap*AT3 (the AT domain from module 3 of the rapamycin PKS), *rap*AT12, *ery*AT1 (the AT domain from module 1 of the erythromycin (DEBS) PKS), or *ery*AT2 coding sequences. Each of these constructs provides a PKS that produces the 13-desmethoxy-13-methyl derivative, except for the rapAT12 replacement, which provides the 13-desmethoxy derivative, i.e., it has a hydrogen where the other derivatives have methyl.

Figure 7 shows the process used to generate the AT replacement constructs. First, a fragment of ~4.5 kb containing module 8 coding sequences from the FK-520 cluster of ATCC 14891 was cloned using the convenient restriction sites SacI and SphI (Step A in Figure 7). The choice of restriction sites used to clone a 4.0 - 4.5 kb fragment comprising module 8 coding sequences from other FK-520 or FK-506 clusters can be different depending on the DNA sequence, but the overall scheme is identical. The unique SacI and SphI restriction sites at the ends of the FK-520 module 8 fragment were then changed to unique Bgl II and NsiI sites by ligation to synthetic linkers (described in

the preceding Examples, see Step B of Figure 7). Fragments containing sequences 5' and 3' of the AT8 sequences were then amplified using primers, described above, that introduced either an *Avr*II site or an *Nhe*I site at two different KS/AT boundaries and an *Aho*I site at the AT DH boundary (Step C of Figure 7). Heterologous AT domains from the rapamycin and erythromycin gene clusters were amplified using primers, as described above, that introduced the same sites as just described (Step D of Figure 7). The fragments were ligated to give hybrid modules with in-frame fusions at the KS/AT and AT/DH boundaries (Step E of Figure 7). Finally, these hybrid modules were ligated into the *Bam*HI and *Pst*I sites of the KC515 vector. The resulting recombinant phage were used to transform the FK-506 and FK-520 producer strains to yield the desired recombinant cells, as described in the preceding Examples.

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The following table shows the location and sequences surrounding the engineered site of each of the heterologous AT domains employed. The FK-506 hybrid construct was used as a control for the FK-520 recombinant cells produced, and a similar FK-520 hybrid construct was used as a control for the FK-506 recombinant cells.

Heterologous AT	Enzyme	Location of Engineered Site
FK-506 AT8	AvrII	GGCCGT <u>bugbab</u> CGTBCGGCGGTCTCGTCGTTC
(hydroxymalonyl)		GRPRRAAVSSF
, ,	NheI	ACCCAGCATCCCSCGATGGGTGAGCG <u>actagc</u> C
	, , , , , , , , , , , , , , , , , , , ,	TQHPAMGERLA
	,	TACGCCTTCCAGCGGCGCCCTACTGGátcgag
	1.7101	YAFQRRPYWIE
rapamycin AT3	AvrII	GACCGGgagggtCCGGGCCGGGCGTCCTTC
(methylmalonyl)	1	D R P P R A G V S S F
	` Nhel	TGGCAGTGGGGGATGGGCAGTGCcctgcqG
		WQWLGMGSALR
	V7 1	TACGCCTTCCAACACCAGCGGTACTGGgtegag
	Xhol	Y A F Q H Q R Y W V E
rapamycin AT12	AvrII	GGCCGAgagagaGGGGGCGTGTCGTCCTTC
(malonyl)		G R A F R A G V S S F
	NheI	TCGCAGCGTGCTGGCATG3GTGAGGAactggcC
ŧ		S Q K A G M G E E L A
	XhoI	TACGCCTTCCAGCACCAGCGCTACTGGctcgag
DEDC .m.		Y A F Q H Q R Y W L E
DEBS AT1	AvrII	GCGCGAuguageCGGGGCGGGGTCTCGTCGTTC
(methylmalonyl)		ARPRRAGVSSF
	Nhel	TGGCAGTGGGCGGGCATGGCCGTCGAcctgctC
•		W Q W A G M A V D L L
	XhoI	TACCCGTTCCAGCGCGAGCGCGTCTGGctcgaa
DEDC ATTS		Y P F Q R E R V W L E
DEBS AT2	AvrIl	GACGGGgtgggCAGGTGTCGGCGTTC
(methylmalonyl)	!	D G V R R A G V S A F
		GCCCAGTGGGAAGGCATGGCGCGGGAattattG

NheI	A	Q	W	Ε	G	М	A	R	E	L	L
	TAT	CCT	TTC	CAG	GGC.	AAG	CGG	TTC	TGG	cla	cta
Xhol	Y	P	F	Q	G	K	R	F	W	L	L

The sequences shown below provide the location of the KS/AT boundaries chosen in the FK-520 module 8 coding sequences. Regions where *AvrII* and *NheI* sites were engineered are indicated by lower case and underlining.

cccsccccatesAncrecTents, bbecsccccategoccaAsAccaAccaGocacggC A S A V E L L T S A S F W P E T D R P R 5 GTGCCGCCCTCCTCCTCCTTCGCCCCTGAGCCGCCAACGCCCAACGCCCACGTCATCCTGGAGGCCG R A A V S S F G V S G T N A H V I GACCGCTAACGGAGACCCCCCGCGGCATCGCCTTCCGGTGACCTTCCCCCTGCTGGTGTCGG G P V T E T P A A S P S G D L P L L V S 10 CACGCTCACCGGAAGCGCTCGACGAGCAGATCCGCCGACTGCGCGCCTACCTGGACACCA A R S P E A L D E Q I R R L R A Y L D T CCCCGGACGTCGACCGGGTGGCCGTGGCACAGACGCTGGCCCGGGCGCACACACTTCGCCC T P D V D R V A V A Q T L A R R T H F A 15 H R A V L L G D T V I T T P P A D R P D AMOTOGTOTTOGTOTACTOCGGGGGGAGGGAGGAGGATC GGATGGGGGAGCAGCAG ELVFVYSGQGTQHPAMGEQL cCGCCGCCCATCCCGTGTTCGCCGACGCCTGGCATGAAGCGCTCCGCCGCCTTGACAACC AAAFPVFADAWHEALRRLDN 20

The sequences shown below provide the location of the AT/DH boundary chosen in the FK-520 module 8 coding sequences. The region where an XhoI site was

TCCTCGGGGCTGGGTCACGGCACGACGCGGATGTGCCCGCGTACGGGTTCCAACGGCGGC

I L G A G S R H D A D V P A Y A F Q R R ACTACTGGatcgagTCGGCACGCCGGCCGCATCCGACGCGGGCCACCCCGTGCTGGGCT H Y W I E S A R P A A S D A G H P V L G

engineered is indicated by lower case and underlining.

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The sequences shown below provide the location of the KS/AT boundaries chosen in the FK-506 module 8 coding sequences. Regions where *AvrII* and *NheI* sites were engineered are indicated by lower case and underlining.

TCGGCCAGGCCGTGGCCGCGACCGGCCGTgcgcgcCGTGCGGCGGTCTCGTCGTTCGGC SARPWPRTGRPRAAVSSFG GTGAGCGGCACCACGCCCACATCATCCTGGAGGCCGGACCGGACCAGGAGGAGCCGTCG 35 V S G T N A H I I L E A G P D Q E E P S GCAGAACCGGCCGGTGACCTCCCGCTGCTCGTGTCGGCACGGTCCCCGGAGGCACTGGAC A E P A G D L P L L V S A R S P E A L D GAGCAGATCGGGCGCCTGCGCGACTATCTCGAUGCCGCCCCGGCGTGGACCTGGCGGCC EOIGRLRDYLDAAPGVDLAA 40 GTGGCGCGGACACTGGCCACGCGTACGCACTTCTCCCACCGCGCCGTACTGCTCGGTGAC V A R T L A T R T H F S H R A V L L G D ACCGTCATCACCGCTCCCCCCGTGGAACAGCCGGGCGAGCTCGTCTTCGTCTACTCGGGA T V I T A P P V E Q P G E L V F V Y S G 45 Q G T O H P A M G E R L A A A F P V F A GACCCGGACGTACCCGCCTACGCCTTCCAGCGGCGGCCCTACTGGATCGAGTCCGCGCCCG D P D V P A Y A F Q R R P Y W I E S A P

The sequences shown below provide the location of the AT/DH boundary chosen in the FK-506 module 8 coding sequences. The region where an *XhoI* site was engineered is indicated by lower case and underlining.

Example 4

Replacement of Methoxyl with Hydrogen or Methyl at C-15 of FK-506 and FK-520

The methods and reagents of the present invention also provide novel FK-506

and FK-520 derivatives in which the methoxy group at C-15 is replaced by a hydrogen or methyl. These derivatives are produced in recombinant host cells of the invention that express recombinant PKS enzymes the produce the derivatives. These recombinant PKS enzymes are prepared in accordance with the methodology of Examples 1 and 2, with the exception that AT domain of module 7, instead of module 8, is replaced. Moreover, the present invention provides recombinant PKS enzymes in which the AT domains of both modules 7 and 8 have been changed. The table below summarizes if e various compounds provided by the present invention.

	Compound	C-13	C-15	Derivative Provided
15	FK-506	hydrogen	hydrogen	13, 15-didesmethoxy-FK-506
	FK-506	hydrogen	methoxy	13-desmethoxy-FK-506
	FK-506	hydrogen	methyl	13,15-didesmethoxy-15-methyl-FK-506
	FK-506	methoxy	hydrogen	15-desmethoxy-FK-506
	FK-506	methoxy	methoxy	Original Compound FK-506
20	FK-506	methoxy	methyl	15-desmethoxy-15-methyl-FK-506
	FK-506	methyl	hydrogen	13,15-didesmethoxy-13-methyl-FK-506
	FK-506	methyl	methoxy	13-desmethoxy-13-methyl-FK-506
	FK-506	methyl	methyl	13,15-didesmethoxy-13,15-dimethyl-FK-506
	FK-520	hydrogen	hydrogen	13, 15-didesmethoxy FK-520
25	FK-520	hydrogen	methoxy	13-desmethoxy FK-520
	FK-520	hydrogen	methyl	13,15-didesmethoxy-15-methyl-FK-520
	FK-520	methoxy	hydrogen	15-desmethoxy-FK-520
	FK-520	methoxy	methoxy	Original Compound FK-520
	FK-520	methoxy	methyl	15-desmethoxy-15-methyl-FK-520
30	FK-520	methyl	hydrogen	13,15-didesmethoxy-13-methyl-FK-520
	FK-520	methyl	methoxy	13-desmethoxy-13-methyl-FK-520
	FK-520	methyl	methyl	13,15-didesmethoxy-13,15-dimethyl-FK-520

Example 5

Replacement of Methoxyl with Ethyl at C-13 and/or C-15 of FK-506 and FK-520

The present invention also provides novel FK-506 and FK-520 derivative compounds in which the methoxy groups at either or both the C-13 and C-15 positions are instead ethyl groups. These compounds are produced by novel PKS enzymes of the invention in which the AT domains of modules 8 and/or 7 are converted to ethylmalonyl specific AT domains by modification of the PKS gene that encodes the module. Ethylmalonyl specific AT domain coding sequences can be obtained from, for example, the FK-520 PKS genes, the niddamycin PKS genes, and the tylosin PKS genes. The novel PKS genes of the invention include not only those in which either or both of the AT domains of modules 7 and 8 have been converted to ethylmalonyl specific AT domain and the other is converted to a malonyl specific or a methylmalonyl specific AT domain.

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Example 6

Neurotrophic Compounds

The compounds described in Examples 1 - 4, inclusive have immunosuppressant activity and can be employed as immunosuppressants in a manner and in formulations similar to those employed for FK-506. The compounds of the invention are generally effective for the prevention of organ rejection in patients receiving organ transplants and in particular can be used for immunosuppression following orthotopic liver transplantation. These compounds also have pharmacokinetic properties and metabolism that are more advantageous for certain applications relative to those of FK-506 or FK-520. These compounds are also neurotrophic; however, for use as neurotrophins, it is desirable to modify the compounds to diminish or abolish their immunosuppressant activity. This can be readily accomplished by hydroxylating the compounds at the C-18 position using established chemical methodology or novel FK-520 PKS genes provided by the present invention.

Thus, in one aspect, the present invention provides a method for stimulating nerve growth that comprises administering a therapeutically effective dose of 18-hydroxy-FK-520. In another embodiment, the compound administered is a C-18,20-dihydroxy-FK-520 derivative. In another embodiment, the compound administered is a C-13-desmethoxy and/or C-15-desmethoxy 18-hydroxy-FK-520 derivative. In another embodiment, the compound administered is a C-13-desmethoxy and/or C-15-desmethoxy 18,20-dihydroxy-FK-520 derivative. In other embodiments, the compounds are the corresponding analogs of FK-506. The 18-hydroxy compounds of the invention

can be prepared chemically, as described in U.S. Patent No. 5,189,042, incorporated herein by reference, or by fermentation of a recombinant host cell provided by the present invention that expresses a recombinant PKS in which the module 5 DH domain has been deleted or rendered non-functional.

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The chemical methodology is as follows. A compound of the invention (~200 mg) is dissolved in 3 mL of dry methylene chloride and added to 45 μL of 2,6-lutidine, and the mixture stirred at room temperature. After 10 minutes, tert-butyldimethylsilyl trifluoromethanesulfonate (64 µL) is added by syringe. After 15 minutes, the reaction mixture is diluted with ethyl acetate, washed with saturated bicarbonate, washed with brine, and the organic phase dried over magnesium sulfate. Removal of solvent in vacuo and flash chromatography on silica gel (ethyl acetate: hexane (1:2) plus 1% methanol) gives the protected compound, which is dissolved in 95% ethanol (2.2 mL) and to which is added 53 uL of pyridine, followed by selenium dioxide (58 mg). The flask is fitted with a water condenser and heated to 70°C on a mantle. After 20 hours, the mixture is cooled to room temperature, filtered through diatomaceous earth, and the filtrate poured into a saturated sodium bicarbonate solution. This is extracted with ethyl acetate, and the organic phase is washed with brine and dried over magnesium sulfate. The solution is concentrated and purified by flash chromatography on silica gel (ethyl acetate: hexane (1:2) plus 1% methanol) to give the protected 18-hydroxy compound. This compound is dissolved in acetonitrile and treated with aqueous HF to remove the protecting groups. After dilution with ethyl acetate, the mixture is washed with saturated bicarbonate and brine, dried over magnesium sulfate, filtered, and evaporated to yield the 18-hydroxy compound. Thus, the present invention provides the C-1S-hydroxyl derivatives of the compounds described in Examples 1 - 4.

Those of skill in the art will recognize that other suitable chemical procedures can be used to prepare the novel 18-hydroxy compounds of the invention. See, e.g., Kawai et al., Jan. 1993, Structure-activity profiles of macrolactam immunosuppressant FK-506 analogues, FEBS Letters 316(2): 107-113. incorporated herein by reference. These methods can be used to prepare both the C18-[S]-OH and C18-[R]-OH enantiomers, with the R enantiomer showing a somewhat lower IC₅₀, which may be preferred in some applications. See Kawai et al., supra. Another preferred protocol is described in Umbreit and Sharpless, 1977, JACS 99(16): 1526-28, although it may be preferable to use 30 equivalents each of SeO₂ and t-BuOOH rather than the 0.02 and 3-4 equivalents, respectively, described in that reference.

All scientific and patent publications referenced herein are hereby incorporated by reference. The invention having now been described by way of written description and example, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments, that the foregoing description and example is for purposes of illustration and not limitation of the following claims.

Claims

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1. An isolated nucleic acid that encodes a CoA ligase, a non-ribosomal peptide synthetase, or a domain of an extender module of a polyketide synthase enzyme that synthesizes FK-520.

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2. The isolated nucleic acid of claim 1 that encodes an extender module, said module comprising a ketosynthase domain, an acyl transferase domain, and an acyl carrier protein domain.

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3. The isolated nucleic acid of claim 1 that encodes an open reading frame said open reading frame comprising coding sequenc... for two or more extender modules, each extender module comprising a ketosynthase domain, an acyl transferase domain, and an acyl carrier protein domain.

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4. The isolated nucleic acid of claim 1 that encodes a gene cluster, said gene cluster comprising two or more open reading frames, each of said open reading frames comprising coding sequences for two or more extender modules, each of said extender modules comprising a ketosynthase domain, an acyl transferase domain, and an acyl carrier protein domain.

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5. The isolated nucleic acid of claim 2, wherein at least one of said domains is a domain of a module of a non-FK-520 polyketide synthase.

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6. The isolated nucleic acid of claim 1, wherein said nucleic acid is a recombinant vector capable of replication in or integration into the chromosome of a host cell.

7. The isolated nucleic acid of claim 6 that is selected from the group consisting

of cosmid pKOS034-120, cosmid pKOS034-124, cosmid pKOS065-M27, and cosmid pKOS065-M21.

8. The isolated nucleic acid of claim 5, wherein said non-FK-520 polyketide synthase is rapamycin polyketide synthase, FK-506 polyketide synthase, or erythromcyin polyketide synthase.

9. A method of preparing a polyketide, said method comprising transforming a host cell with a recombinant DNA vector of claim 6, and culturing said host cell under conditions such that said polyketide synthase is produced and catalyzes synthesis of said polyketide.

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- 10. The method of claim 9, wherein said host cell is a Streptomyces host cell.
- 11. The method of claim 9, wherein said polyketide is selected from the group consisting of FK-520, 13-desmethoxy-FK-520, and 13-desmethoxy-FK-506.

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- 12. A recombinant host cell that expresses a recombinant polyketide synthase selected from the group consisting of: (i) an FK-520 polyketide synthase in which at least one AT domain is replaced by an AT domain of a non-FK-520 polyketide synthase; (ii) an FK-506 polyketide synthase in which at least one AT domain is replaced by an AT domain of a non-FK-506 polyketide synthase; (iii) an FK-520 polyketide synthase in which at least one DH domain has been deleted; (iv) an FK-506 polyketide synthase in which at least one DH domain has been deleted.
- 13. The recombinant host cell of claim 12 that expresses an FK-520 polyketide synthase in which an AT domain of module 8 has been replaced by an AT domain that binds malonyl CoA, methylmalonyl CoA, or ethylmalonyl CoA.
 - 14. The recombinant host cell of claim 12 that expresses an FK-506 polyketide synthase in which an AT domain of module 8 has been replaced by an AT domain that binds malonyl CoA, methylmalonyl CoA, or ethylmalonyl CoA.
 - 15. The recombinant host cell of claim 13, wherein a DH domain of module 5 or module 6 has been deleted.
- 30 16. The recombinant host cell of claim 14, wherein a DH domain of module 5 or module 6 has been deleted.
 - 17. A recombinant host cell that comprises recombinant genes coding for enzymes sufficient for synthesis of ethylmalonyl CoA or 2-hydroxymalonyl CoA.

18. A polyketide having the structure

- wherein, R₁ is hydrogen, methyl, ethyl, or allyl; R₂ is hydrogen or hydroxyl, provided that when R₂ is hydrogen, there is a double bond between C-20 and C-19; R₃ is hydrogen or hydroxyl; R₄ is methoxyl, hydrogen, methyl, or ethyl; and R₅ is methoxyl, hydrogen, methyl, or ethyl; but not including FK-506, FK-520, 18-hydroxy-FK-520, and 18-hydroxy-FK-506.
- 19. The polyketide of claim 18 that is 13-desmethoxy-FK-506.
 - 20. The polyketide of claim 18 that is 13-desmethoxy-18-hydroxy-FK-520.

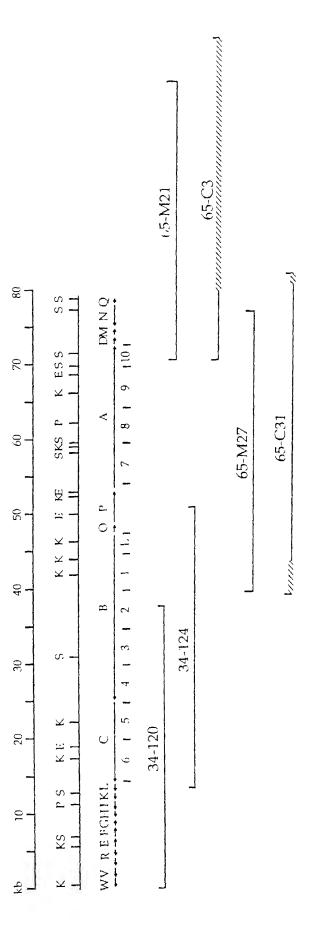


Figure 1

Figure 2

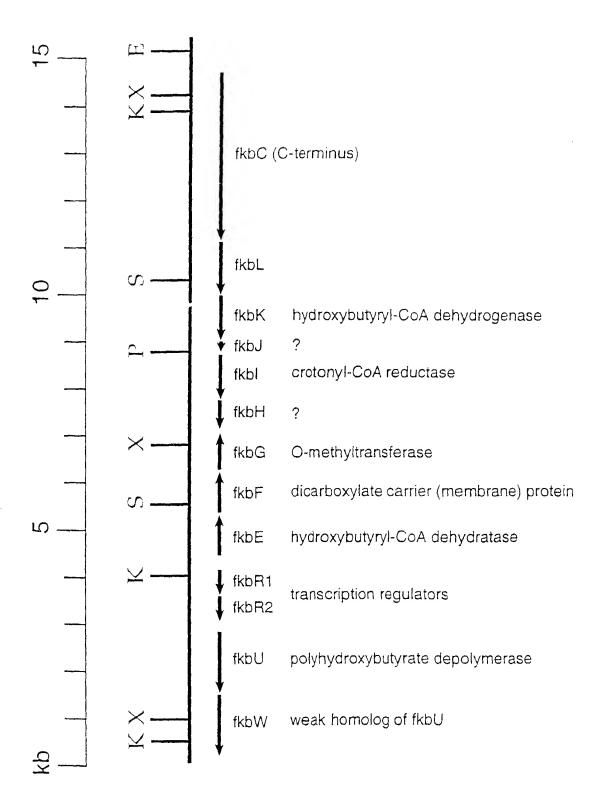


Figure 3

Figure 4

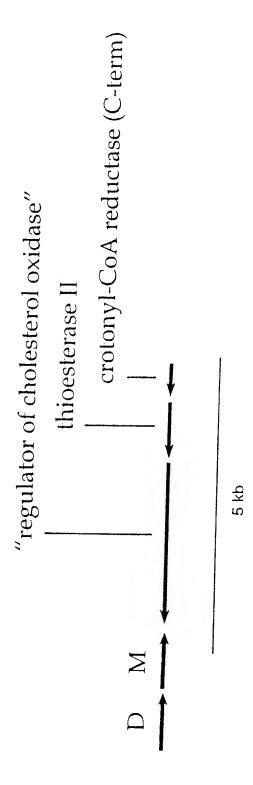


Figure 5

Figure 6

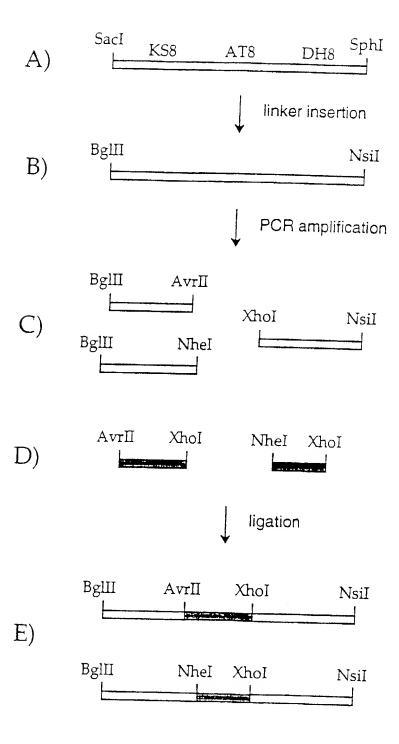


Figure 7

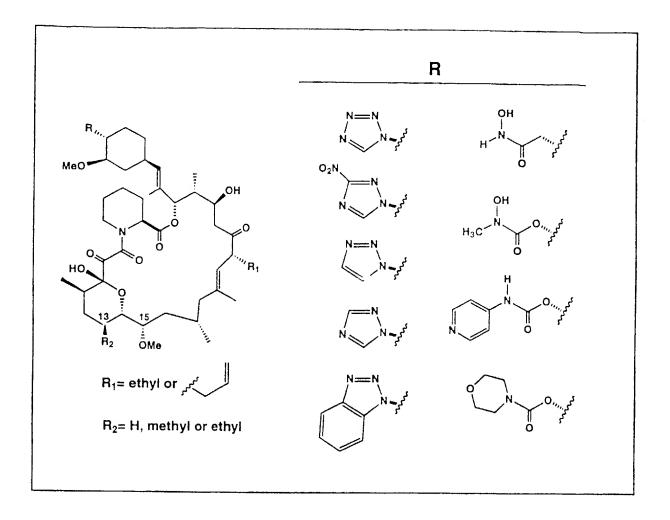


Figure 8 Part A

$$R_{1} = \text{ethyl or ethyl}$$

$$R_{2} = H, \text{ methyl or ethyl}$$

$$R_{3} = H, \text{ methyl or ethyl}$$

$$R_{4} = \text{ethyl or ethyl}$$

$$R_{5} = H, \text{ methyl or ethyl or$$



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7:

C12N 15/52, 15/54, 15/62, 9/10, C12P 17/18, 19/32, C07D 498/18 // (C07D 498/18, 311:00, 273:00, 211:00)

(11) International Publication Number:

WO 00/20601

(43) International Publication Date:

13 April 2000 (13.04.00)

(21) International Application Number:

PCT/US99/22886

A2

(22) International Filing Date:

1 October 1999 (01.10.99)

(30) Priority Data:

US 60/102,748 2 October 1998 (02.10.98) US 60/123,810 11 March 1999 (11.03.99) 60.139,650 17 June 1999 (17.06.99) US

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(81) Designated States: AL, AM, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, IL, IS, JP, KG, KP, KR, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TV UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, IJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, R, IE, IT, LU, MC, NL, 117, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG:

Without interactional search report and to be republished upon receipt of that report

With an indication in relation to deposited biological material furnished under Rule 13bis separately from the description

(54) Title: POLYKETIDE SYNTHASE ENZYMES AND RECOMBINANT DNA CONSTRUCTS THEREFOR

(57) Abstract

Host cells comprising recombinant vectors encoding the FK-520 polyketide synthase and FK-520 modification enzymes can be used to produce the FK-520 polyketide. Recombinant DNA constructs comprising one or more FK-520 polyketide synthase domains, modules, open reading frames, and variants thereof can be used to produce recombinant polyketide synthases and a variety of different polyketides with application as pharmaceutical and veterinary products.

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POLYKETIDE SYNTHASE ENZYMES AND RECOMBINANT DNA CONSTRUCTS THEREFOR

Field of the Invention

The present invention relates to polyketides and the polyketide synthase (PKS) enzymes that produce them. The invention also relates generally to genes encoding PKS enzymes and to recombinant host cells containing such genes and in which expression of such genes leads to the production of polyketides. The present invention also relates to compounds useful as medicaments having immunosuppressive and/or neurotrophic activity. Thus, the invention relates to the fields of chemistry, molecular biology, and agricultural, medical, and veterinary technology.

Background of the Invention

Polyketides are a class of compounds synthesized from 2-carbon units through a series of condensations and subsequent modifications. Polyketides occur in many types of organisms, including fungi and mycelial bacteria, in particular, the actinomycetes. Polyketides are biologically active molecules with a wide variety of structures, and the class encompasses numerous compounds with diverse activities. Tetracycline, erythromycin, epothilone, FK-506, FK-520, narbomycin, picromycin, rapamycin, spinocyn, and tylosin are examples of polyketides. Given the difficulty in producing polyketide compounds by traditional chemical methodology, and the typically low production of polyketides in wild-type cells, there has been considerable interest in finding improved or alternate means to produce polyketide compounds.

This interest has resulted in the cloning, analysis, and manipulation by recombinant DNA technology of genes that encode PKS enzymes. The resulting technology allows one to manipulate a known PKS gene cluster either and roduce the polyketide synthesized by that PKS at higher levels than occur in nature or in hosts that otherwise do not produce the polyketide. The technology also allows one to produce molecules that are structurally related to, but distinct from, the polyketides produced from known PKS gene clusters. See, e.g., PCT publication Nos. WO 93/13663; 95/08548; 96/40968; 97/02358; 98/27203; and 98/49315; United States Patent Nos. 4,874,748; 5.063,155; 5.098,837; 5,149,639; 5,672,491; 5,712,146; 5,830,750; and 5.843,718; and Fu et al., 1994, Biochemistry 33:

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9321-9326; McDaniel et al., 1993, Science 262: 1546-1550; and Rohr, 1995, Angew. Chem. Int. Ed. Engl. 34(8): 881-888, each of which is incorporated herein by reference.

Polyketides are synthesized in nature by PKS enzymes. These enzymes, which are complexes of multiple large proteins, are similar to the synthases that catalyze condensation of 2-carbon units in the biosynthesis of fatty acids. PKSs catalyze the biosynthesis of polyketides through repeated, decarboxylative Claisen condensations between acylthioester building blocks. The building blocks used to form complex polyketides are typically acylthioesters, such as acetyl, butyryl, propionyl, malonyl, hydroxymalonyl, methylmalonyl, and ethylmalonyl CoA. Other building blocks include amino acid like acylthioesters. PKS enzymes that incorporate such building blocks include an activity that functions as an amino acid ligase (an AMP ligase) or as a non-ribosomal peptide synthetase (NRPS). Two major types of PKS enzymes are known; these differ in their composition and mode of synthesis of the polyketide synthesized. These two major types of PKS enzymes are commonly referred to as Type I or "modular" and Type II "iterative" PKS enzymes.

In the Type I or modular PKS enzyme group, a set of separate catalytic active sites (each active site is termed a "domain", and a set thereof is termed a "module") exists for each cycle of carbon chain elongation and modification in the polyketide synthesis pathway. The typical modular PKS is composed of several large polypeptides, which can be segregated from amino to carboxy termini into a loading module, multiple extender modules, and a releasing (or thioesterase) domain. The PKS enzyme known as 6-deoxyerythronolide B synthase (DEBS) is a Type I PKS. In DEBS, there is a loading module, six extender modules, and a thioesterase (TE) domain. The loading module, six extender modules, and TE of DEBS are present on three separate proteins (designated DEBS-1, DEBS-2, and DEBS-3, with two extender modules per protein). Each of the DEBS polypeptides is encoded by a separate open reading frame (ORF) or gene; these genes are known as eryAI, eryAII, and eryAIII. See Caffrey et al., 1992, FEBS Letters 304: 205, and U.S. Patent No. 5,824,513, each of which is incorporated herein by reference.

Generally, the loading module is responsible for binding the first building block used to synthesize the polyketide and transferring it to the first extender module. The loading module of DEBS consists of an acyltransferase (AT) domain and an acyl carrier protein (ACP) domain. Another type of loading module utilizes an inactivated ketosynthase (KS) domain and AT and ACP domains. This inactivated KS is in some instances called KS^Q, where the superscript letter is the abbreviation for the amino acid, glutamine, that is

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present instead of the active site cysteine required for ketosynthase activity. In other PKS enzymes, including the FK-506 PKS, the loading module incorporates an unusual starter unit and is composed of a CoA ligase like activity domain. In any event, the loading module recognizes a particular acyl-CoA (usually acetyl or propionyl but sometimes butyryl or other acyl-CoA) and transfers it as a thiol ester to the ACP of the loading module.

The AT on each of the extender modules recognizes a particular extender-CoA (malonyl or alpha-substituted malonyl, i.e., methylmalonyl, ethylmalonyl, and 2-hydroxymalonyl) and transfers it to the ACP of that extender module to form a thioester. Each extender module is responsible for accepting a compound from a prior module, binding a building block, attaching the building block to the compound from the prior module, optionally performing one or more additional functions, and transferring the resulting compound to the next module.

Each extender module of a modular PKS contains a KS, AT, ACP, and zero, one, two, or three domains that modify the beta-carbon of the growing polyketide chain. A typical (non-loading) minimal Type I PKS extender module is exemplified by extender module three of DEBS, which contains a KS domain, an AT domain, and an ACP domain. These three domains are sufficient to activate a 2-carbon extender unit and attach it to the growing polyketide molecule. The next extender module, in turn, is responsible for attaching the next building block and transferring the growing compound to the next extender module until synthesis is complete.

Once the PKS is primed with acyl- and malonyl-ACPs, the acyl group of the loading module is transferred to form a thiol ester (trans-esterification) at the KS of the first extender module; at this stage, extender module one possesses an acyl-KS and a malonyl (or substituted malonyl) ACP. The acyl group derived from the loading module is then covalently attached to the alpha-carbon of the malonyl group to form a carbon-carbon bond, driven by concomitant decarboxylation, and generating a new acyl-ACP that has a backbone two carbons longer than the loading building block (elongation or extension).

The polyketide chain, growing by two carbons each extender module, is sequentially passed as covalently bound thiol esters from extender module to extender module, in an assembly line-like process. The carbon chain produced by this process alone would possess a ketone at every other carbon atom, producing a polyketone, from which the name polyketide arises. Most commonly, however, additional enzymatic activities modify the beta

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keto group of each two carbon unit just after it has been added to the growing polyketide chain but before it is transferred to the next module.

Thus, in addition to the minimal module containing KS. AT, and ACP domains necessary to form the carbon-carbon bond, and as noted above, other domains that modify the beta-carbonyl moiety can be present. Thus, modules may contain a ketoreductase (KR) domain that reduces the keto group to an alcohol. Modules may also contain a KR domain plus a dehydratase (DH) domain that dehydrates the alcohol to a double bond. Modules may also contain a KR domain, and an enoylreductase (ER) domain that converts the double bond product to a saturated single bond using the beta carbon as a methylene function. An extender module can also contain other enzymatic activities, such as, for example, a methylase or dimethylase activity.

After traversing the final extender module, the polyketide encounters a releasing domain that cleaves the polyketide from the PKS and typically cyclizes the polyketide. For example, final synthesis of 6-dEB is regulated by a TE domain located at the end of extender module six. In the synthesis of 6-dEB, the TE domain catalyzes cyclization of the macrolide ring by formation of an ester linkage. In FK-506, FK-520, rapamycin, and similar polyketides, the TE activity is replaced by a RapP (for rapamycin) or RapP like activity that makes a linkage incorporating a pipecolate acid residue. The enzymatic activity that catalyzes this incorporation for the rapamycin enzyme is known as RapP, encoded by the *rapP* gene. The polyketide can be modified further by tailoring enzymes: these enzymes add carbohydrate groups or methyl groups, or make other modifications, i.e., oxidation or reduction, on the polyketide core molecule. For example, 6-dEB is hydroxylated at C-6 and C-12 and glycosylated at C-3 and C-5 in the synthesis of erythromycin A.

In Type I PKS polypeptides, the order of catalytic domains is conserved. When all beta-keto processing domains are present in a module, the order of domains in that module from N-to-C-terminus is always KS, AT, DH, ER, KR, and ACP. Some or all of the beta-keto processing domains may be missing in particular modules, but the order of the domains present in a module remains the same. The order of domains within modules is believed to be important for proper folding of the PKS polypetides into an active complex. Importantly, there is considerable flexibility in PKS enzymes, which allows for the genetic engineering of novel catalytic complexes. The engineering of these enzymes is achieved by modifying, adding, or deleting domains, or replacing them with those taken from other Type I PKS enzymes. It is also achieved by deleting, replacing, or adding entire modules with those

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taken from other sources. A genetically engineered PKS complex should of course have the ability to catalyze the synthesis of the product predicted from the genetic alterations made.

Alignments of the many available amino acid sequences for Type I PKS enzymes has approximately defined the boundaries of the various catalytic domains. Sequence alignments also have revealed linker regions between the catalytic domains and at the Nand C-termini of individual polypeptides. The sequences of these linker regions are less well conserved than are those for the catalytic domains, which is in part how linker regions are identified. Linker regions can be important for proper association between domains and between the t lauar polypeptides that comprise the PKS complex. One can thus view the linkers and domains together as creating a scaffold on which the domains and modules are positioned in the correct orientation to be active. This organization and positioning, if retained, permits PKS domains of different or identical substrate specificities to be substituted (usually at the DNA level) between PKS enzymes by various available methodologies. In selecting the boundaries of, for example, an AT replacement, one can thus make the replacement so as to retain the linkers of the recipient PKS or to replace them with the linkers of the donor PKS AT domain, or, preferably, make both constructs to ensure that the correct linker regions between the KS and AT domains have been included in at least one of the engineered enzymes. Thus, there is considerable flexibility in the design of new PKS enzymes with the result that known polyketides can be produced more effectively, and novel polyketides useful as pharmaceuticals or for other purposes can be made.

By appropriate application of recombinant DNA technology, a wide variety of polyketides can be prepared in a variety of different host cells provided one has access to nucleic acid compounds that encode PKS proteins and polyketide modification enzymes. The present invention helps meet the need for such nucleic acid compounds by providing recombinant vectors that encode the FK-520 PKS enzyme and various FK-520 modification enzymes. Moreover, while the FK-506 and FK-520 polyketides have many useful activities, there remains a need for compounds with similar useful activities but with better pharmacokinetic profile and metabolism and fewer side-effects. The present invention helps meet the need for such compounds as well.

Summary of the Invention

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In one embodiment, the present invention provides recombinant DNA vectors that encode all or part of the FK-520 PKS enzyme. Illustrative vectors of the invention include cosmid pKOS034-120, pKOS034-124, pKOS065-C31, pKOS065-C3, pKOS065-M27, and pKOS065-M21. The invention also provides nucleic acid compounds that encode the various domains of the FK-520 PKS, i.e., the KS, AT, ACP, KR, DH, and ER domains. These compounds can be readily used, alone or in combination with nucleic acids encoding other FK-520 or non-FK-520 PKS domains, as intermediates in the construction of recombinant vectors that encode all or part of PKS enzymes that make novel polyketides.

The invention also provides isolated nucleic acids that encode all or part of one or more modules of the FK-520 PKS, each module comprising a ketosynthase activity, an acyl transferase activity, and an acyl carrier protein activity. The invention provides an isolated nucleic acid that encodes one or more open reading frames of FK-520 PKS genes, said open reading frames comprising coding sequences for a CoA ligase activity, an NRPS activity, or two or more extender modules. The invention also provides recombinant expression vectors containing these nucleic acids.

In another embodiment, the invention provides isolated nucleic acids that encode all or a part of a PKS that contains at least one module in which at least one of the domains in the module is a domain from a non-FK-520 PKS and at least one domain is from the FK-520 PKS. The non-FK-520 PKS domain or module originates from the rapamycin PKS, the FK-506 PKS, DEBS, or another PKS. The invention also provides recombinant expression vectors containing these nucleic acids.

In another embodiment, the invention provides a method of preparing a polyketide, said method comprising transforming a host cell with a recombinant DNA vector that encodes at least one module of a PKS, said module comprising at least one FK-520 PKS domain, and culturing said host cell under conditions such that said PKS is produced and catalyzes synthesis of said polyketide. In one aspect, the method is practiced with a *Streptomyces* host cell. In another aspect, the polyketide produced is FK-520. In another aspect, the polyketide produced is a polyketide related in structure to FK-520. In another aspect, the polyketide produced is a polyketide related in structure to FK-506 or rapamycin.

In another embodiment, the invention provides a set of genes in recombinant form sufficient for the synthesis of ethylmalonyl CoA in a heterologous host cell. These genes and the methods of the invention enable one to create recombinant host cells with the ability to produce polyketides or other compounds that require ethylmalonyl CoA for biosynthesis.

The invention also provides recombinant nucleic acids that encode AT domains specific for ethylmalonyl CoA. Thus, the compounds of the invention can be used to produce polyketides requiring ethylmalonyl CoA in host cells that otherwise are unable to produce such polyketides.

In another embodiment, the invention provides a set of genes in recombinant form sufficient for the synthesis of 2-hydroxymalonyl CoA and 2-methoxymalonyl CoA in a heterologous host cell. These genes and the methods of the invention enable one to create recombinant host cells with the ability to produce polyketides or other compounds that require 2-hydroxymalonyl CoA for biosynth siz. The invention also provides recombinant nucleic acids that encode AT domains specific for 2-hydroxymalonyl CoA and 2-methoxymalonyl CoA. Thus, the compounds of the invention can be used to produce polyketides requiring 2-hydroxymalonyl CoA or 2-methoxymalonyl CoA in host cells that are otherwise unable to produce such polyketides.

In another embodiment, the invention provides a compound related in structure to FK-520 or FK-506 that is useful in the treatment of a medical condition. These compounds include compounds in which the C-13 methoxy group is replaced by a moiety selected from the group consisting of hydrogen, methyl, and ethyl moieties. Such compounds are less susceptible to the main *in vivo* pathway of degradation for FK-520 and FK-506 and related compounds and thus exhibit an improved pharmacokinetic profile. The compounds of the invention also include compounds in which the C-15 methoxy group is replaced by a moiety selected from the group consisting of hydrogen, methyl, and ethyl moieties. The compounds of the invention also include the above compounds further modified by chemical methodology to produce derivatives such as, but not limited to, the C-18 hydroxyl derivatives, which have potent neurotrophin but not immunosuppression activities.

Thus, the invention provides polyketides having the structure:

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wherein, R_1 is hydrogen, methyl, or allyl: R_2 is hydrogen or hydroxyl, provided that when R_2 is hydrogen, there is a double bond between C-20 and C-19; R_3 is hydrogen or hydroxyl: R_4 is methoxyl, hydrogen, methyl, or ethyl; and R_3 is methoxyl, hydrogen, methyl, or ethyl; but not including FK-506, FK-520, 18-hydroxy-FK-520, and 18-hydroxy-FK-506. The invention provides these compounds in purified form and in pharmaceutical compositions.

In another embodiment, the invention provides a method for treating a medical condition by administering a pharmaceutically efficacious dose of a compound of the invention. The compounds of the invention may be administered to achieve immunosuppression or to stimulate nerve growth and regeneration.

These and other embodiments and aspects of the invention will be more fully understood after consideration of the attached Drawings and their brief description below, together with the detailed description, examples, and claims that follow.

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Brief Description of the Drawings

Figure 1 shows a diagram of the FK-520 biosynthetic gene cluster. The top line provides a scale in kilobase pairs (kb). The second line shows a restriction map with selected restriction enzyme recognition sequences indicated. K is *KpnI*; X is *XhoI*, S is *SacI*; P is *PstI*; and E is *EcoRI*. The third line indicates the position of FK-520 PKS and related genes. Genes are abbreviated with a one letter designation, i.e., C is *fkbC*. Immediately under the third line are numbered segments showing where the loading module (L) and ten different extender modules (numbered 1 - 10) are encoded on the various genes shown. At the bottom of the Figure, the DNA inserts of various commids of the invention (i.e., 34-124 is cosmid pKOS034-124) are shown in alignment with the FK-520 biosynthetic gene cluster.

Figure 2 shows the loading module (load), the ten extender modules, and the peptide synthetase domain of the FK-520 PKS, together with, on the top line, the genes that encode the various domains and modules. Also shown are the various intermediates in FK-520 biosynthesis, as well as the structure of FK-520, with carbons 13, 15, 21, and 31 numbered. The various domains of each module and subdomains of the loading module are also shown. The darkened circles showing the DH domains in modules 2, 3, and 4 indicate that the dehydratase domain is not functional as a dehydratase; this domain may affect the

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stereochemistry at the corresponding position in the polyketide. The substituents on the FK-520 structure that result from the action of non-PKS enzymes are also indicated by arrows, together with the types of enzymes or the genes that code for the enzymes that mediate the action. Although the methyltransferase is shown acting at the C-13 and C-15 hydroxyl groups after release of the polyketide from the PKS, the methyltransferase may act on the 2-hydroxymalonyl substrate prior to or contemporaneously with its incorporation during polyketide synthesis.

Figure 3 shows a close-up view of the left end of the FK-520 gene cluster, which contains at least ten additional gw ies. The ethyl side chair, on carbon 21 of FK-520 (Figure 2) is derived from an ethylmalonyl CoA extender unit that is incorporated by an ethylmalonyl specific AT domain in extender module 4 of the PKS. At least four of the genes in this region code for enzymes involved in ethylmalonyl biosynthesis. The polyhydroxybutyrate depolymerase is involved in maintaining hydroxybutyryl-CoA pools during FK-520 production. Polyhydroxybutyrate accumulates during vegetative growth and disappears during stationary phase in other *Streptomyces* (Ranade and Vining, 1993, *Can. J. Microbiol.* 39:377). Open reading frames with unknown function are indicated with a question mark.

Figure 4 shows a biosynthetic pathway for the biosynthesis of ethylmalonyl CoA from acetoacetyl CoA consistent with the function assigned to four of the genes in the FK-520 gene cluster shown in Figure 3.

Figure 5 shows a close-up view of the right-end of the FK-520 PKS gene cluster (and of the sequences on cosmid pKOS065-C31). The genes shown include *fkbD*, *fkbM* (a methyl transferase that methylates the hydroxyl group on C-31 of FK-520), *fkbN* (a homolog of a gene described as a regulator of cholesterol oxidase and that is believed to be a transcriptional activator), *fkbQ* (a type II thioesterase, which can increase polyketide production levels), and *fkbS* (a crotonyl-CoA reductase involved in the biosynthesis of ethylmalonyl CoA).

Figure 6 shows the proposed degradative pathway for tacrolimus (FK-506) metabolism.

Figure 7 shows a schematic process for the construction of recombinant PKS genes of the invention that encode PKS enzymes that produce 13-desmethoxy FK-506 and FK-520 polyketides of the invention, as described in Example 4, below.

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Figure 8, in Parts A and B, shows certain compounds of the invention preferred for dermai application in Part A and a synthetic route for making those compounds in Part B.

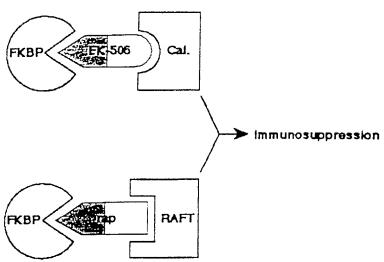
Detailed Description of the Invention

Given the valuable pharmaceutical properties of polyketides, there is a need for methods and reagents for producing large quantities of polyketides, as well as for producing related compounds not found in nature. The present invention provides such methods and reagents, with particular application to methods and reagents for producing the polyketides known as FK-520, also known as ascomycin or L-683,590 (see ! lit et al., 1993, JACS 115:9925), and FK-506, also known as tacrolimus. Tacrolimus is a macrolide immunosuppressant used to prevent or treat rejection of transplanted heart, kidney, liver, lung, pancreas, and small bowel allografts. The drug is also useful for the prevention and treatment of graft-versus-host disease in patients receiving bone marrow transplants, and for the treatment of severe, refractory uveitis. There have been additional reports of the unapproved use of tacrolimus for other conditions, including alopecia universalis, autoimmune chronic active hepatitis, inflammatory bowel disease, multiple sclerosis, primary biliary cirrhosis, and scleroderma. The invention provides methods and reagents for making novel polyketides related in structure to FK-520 and FK-506, and structurally related polyketides such as rapamycin.

The FK-506 and rapamycin polyketides are potent immunosuppressants, with chemical structures shown below.

FK-520 differs from FK-506 in that it lacks the allyl group at C-21 of FK-506, having instead an ethyl group at that position, and has similar activity to FK-506, albeit reduced immunosuppressive activity.

These compounds act through initial formation of an intermediate complex with protein "immunophilins" known as FKBPs (FK-506 binding proteins), including FKBP-12. Immunophilins are a class of cytosolic proteins that form complexes with molecules such as FK-506, FK-520, and rapamycin that in turn serve as ligands for other cellular targets involved in signal transduction. Binding of FK-506, FK-520, and rapamycin to FKBP occurs through the structurally similar segments of the polyketide molecules, known as the "FKBP-binding domain" (as generally but not precisely indicated by the stippled regions in the structures above). The FK-506-FKBP complex then binds calcineurin, while the rapamycin-FKBP complex binds to a protein known as RAFT-1. Binding of the FKBP-polyketide complex to these second proteins occurs through the dissimilar regions of the drugs known as the "effector" domains.



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The three component FKBP-polyketide-effector complex is required for signal transduction and subsequent immunosuppressive activity of FK-506. FK-520, and rapamycin. Modifications in the effector domains of FK-506. FK-520, and rapamycin that destroy binding to the effector proteins (calcineurin or RAFT) lead to loss of immunosuppressive activity, even though FKBP binding is unaffected. Further, such analogs antagonize the immunosuppressive effects of the parent polyketides, because they compete for FKBP. Such non-immunosuppressive analogs also show reduced toxicity (see Dumont et al., 1992, Journal of Experimental Medicine 176, 751-760), indicating that much of the toxicity of these drugs is not linked to FKBP binding.

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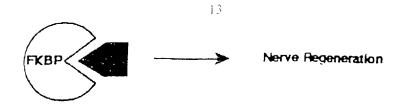
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In addition to immunosuppressive activity, FK-520. FK-506, and rapamycin have neurotrophic activity. In the central nervous system and in peripheral nerves, immunophilins are referred to as "neuroimmunophilins". The neuroimmunophilin FKBP is markedly enriched in the central nervous system and in peripheral nerves. Molecules that bind to the neuroimmunophilin FKBP, such as FK-506 and FK-520, have the remarkable effect of stimulating nerve growth. *In vitro*, they act as neurotrophins, i.e., they promote neurite outgrowth in NGF-treated PC12 cells and in sensory neuronal cultures, and in intact animals, they promote regrowth of damaged facial and sciatic nerves, and repair iesioned serotonin and dopamine neurons in the brain. See Gold *et al.*, Jun. 1999, *J. Pharm. Exp. Ther. 289*(3): 1202-1210; Lyons *et al.*, 1994, *Proc. National Academy of Science 91*: 3191-3195; Gold *et al.*, 1995, *Journal of Neuroscience 15*: 7509-7516; and Steiner *et al.*, 1997, *Proc. National Academy of Science 94*: 2019-2024. Further, the restored central and peripheral neurons appear to be functional.

Compared to protein neurotrophic molecules (BNDF, NGF, etc.), the small-molecule neurotrophins such as FK-506, FK-520, and rapamycin have different, and often advantageous, properties. First, whereas protein neurotrophins are difficult to deliver to their intended site of action and may require intra-cranial injection, the small-molecule neurotrophins display excellent bioavailability; they are active when administered subcutaneously and orally. Second, whereas protein neurotrophins show quite specific effects, the small-molecule neurotrophins show rather broad effects. Finally, whereas protein neurotrophins often show effects on normal sensory nerves, the small-molecule neurotrophins do not induce aberrant sprouting of normal neuronal processes and seem to affect damaged nerves specifically. Neuroimmunophilin ligands have potential therapeutic utility in a variety of disorders involving nerve degeneration (e.g. multiple sclerosis, Parkinson's disease, Alzheimer's disease, stroke, traumatic spinal cord and brain injury, peripheral neuropathies).

Recent studies have shown that the immunosuppressive and neurite outgrowth activity of FK-506, FK-520, and rapamycin can be separated; the neuroregenerative activity in the absence of immunosuppressive activity is retained by agents which bind to FKBP but not to the effector proteins calcineurin or RAFT. See Steiner *et al.*, 1997, *Nature Medicine* 3: 421-428.



Available structure-activity data show that the important features for neurotrophic activity of rapamycin, FK-520, and FK-506 lie within the common, contiguous segments of the macrolide ring that bind to FKBP. This portion of the molecule is termed the "FKBP binding domain" (see VanDuyne et al., 1993, Journal of Molecular Biology 229: 105-124.). Nevertheless, the effector domains of the parent macrolides contribute to conformational rigidity of the binding domain and thus indirectly contribute to FKBP binding.

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"FKBP binding domain"

There are a number of other reported analogs of FK-506, FK-520, and rapamycin that bind to FKBP but not the effector protein calcineurin or RAFT. These analogs show effects on nerve regeneration without immunosuppressive effects.

Naturally occurring FK-520 and FK-506 analogs include the antascomycins, which are FK-506-like macrolides that lack the functional groups of FK-506 that bind to calcineurin (see Fehr et al., 1996, *The Journal of Antibiotics 49*: 230-233). These molecules bind FKBP as effectively as does FK-506; they antagonize the effects of both FK-506 and rapamycin, yet lack immunosuppressive activity.

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Antascomyon A

Other analogs can be produced by chemically modifying FK-506, FK-520, or rapamycin. One approach to obtaining neuroimmunophilin ligands is to destroy the effector binding region of FK-506, FK-520, or rapamycin by chemical modification. While the chemical modifications permitted on the parent compounds are quite limited, some useful chemically modified analogs exist. The FK-520 analog L-685,818 (ED₅₀ = 0.7 nM for FKBP binding; see Dumont *et al.*, 1992), and the rapamycin analog WAY-124,466 (IC₅₀ = 12.5 nM; see Ocain *et al.*, 1993, *Biochemistry Biophysical Research Communications 192*: 1340-134693) are about as effective as FK-506, FK-520, and rapamycin at promoting neurite outgrowth in sensory neurons (see Steiner *et al.*, 1997).

One of the few positions of rapamycin that is readily amenable to chemical modification is the allylic 16-methoxy group; this reactive group is readily exchanged by acid-catalyzed nucleophilic substitution. Replacement of the 16-methoxy group of rapamycin with a variety of bulky groups has produced analogs showing selective loss of immunosuppressive activity while retaining FKBP-binding (see Luengo *et al.*, 1995, *Chemistry & Biology 2*: 471-481). One of the best compounds, 1, below, shows complete

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loss of activity in the splenocyte proliferation assay with only a 10-fold reduction in binding to FKBP.

There are also synthetic analogs of FKBP binding domains. These compounds reflect an approach to obtaining neuroimmunophilin ligands based on "rationally designed" molecules that retain the FKBP-binding region in an appropriate conformation for binding to FKBP, but do not possess the effector binding regions. In one example, the ends of the FKBP binding domain were tethered by hydrocarbon chains (see Holt *et al.*, 1993, *Journal of the American Chemical Society 115*: 9925-9938); the best analog, **2**, below, binds to FKBP about as well as FK-506. In a similar approach, the ends of the FKBP binding domain were tethered by a tripeptide to give analog **3**, below, which binds to FKBP about 20-fold poorer than FK-506. These compounds are anticipated to have neuroimmunophilin binding activity.

In a primate MPTP model of Parkinson's disease, administration of FKBP ligand GPI-1046 caused brain cells to regenerate and behavioral measures to improve. MPTP is a neurotoxin, which, when administered to animals, selectively damages nigral-striatal dopamine neurons in the brain, mimicking the damage caused by Parkinson's disease. Whereas, before treatment, animals were unable to use affected limbs, the FKBP ligand

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restored the ability of animals to feed themselves and gave improvements in measures of locomotor activity, neurological outcome, and fine motor control. There were also corresponding increases in regrowth of damaged nerve terminals. These results demonstrate the utility of FKBP ligands for treatment of diseases of the CNS.

From the above description, two general approaches towards the design of non-immunosuppressant, neuroimmunophilin ligands can be seen. The first involves the construction of constrained cyclic analogs of FK-506 in which the FKBP binding domain is fixed in a conformation optimal for binding to FKBP. The advantages of this approach are that the conformation of the analogs can be accurately modeled and predicted by computational methods, and the analogs closely resemble parent molecules that have proven pharmacological properties. A disadvantage is that the difficult chemistry limits the numbers and types of compounds that can be prepared. The second approach involves the trial and error construction of acyclic analogs of the FKBP binding domain by conventional medicinal chemistry. The advantages to this approach are that the chemistry is suitable for production of the numerous compounds needed for such interactive chemistry-bioassay approaches. The disadvantages are that the molecular types of compounds that have emerged have no known history of appropriate pharmacological properties, have rather labile ester functional groups, and are too conformationally mobile to allow accurate prediction of conformational properties.

The present invention provides useful methods and reagents related to the first approach, but with significant advantages. The invention provides recombinant PKS genes that produce a wide variety of polyketides that cannot otherwise be readily synthesized by chemical methodology alone. Moreover, the present invention provides polyketides that have either or both of the desired immunosuppressive and neurotrophic activities, some of which are produced only by fermentation and others of which are produced by fermentation and chemical modification. Thus, in one aspect, the invention provides compounds that optimally bind to FKBP but do not bind to the effector proteins. The methods and reagents of the invention can be used to prepare numerous constrained cyclic analogs of FK-520 in which the FKBP binding domain is fixed in a conformation optimal for binding to FKBP. Such compounds will show neuroimmunophilin binding (neurotrophic) but not immunosuppressive effects. The invention also allows direct manipulation of FK-520 and related chemical structures via genetic engineering of the enzymes involved in the biosynthesis of FK-520 (as well as related compounds, such as FK-506 and rapamycin);

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similar chemical modifications are simply not possible because of the complexity of the structures. The invention can also be used to introduce "chemical handles" into normally inert positions that permit subsequent chemical modifications.

Several general approaches to achieve the development of novel neuroimmunophilin ligands are facilitated by the methods and reagents of the present invention. One approach is to make "point mutations" of the functional groups of the parent FK-520 structure that bind to the effector molecules to eliminate their binding potential. These types of structural modifications are difficult to perform by chemical modification, but can be readily accomplished with the methods and reagents of the invention.

A second, more extensive approach facilitated by the present invention is to utilize molecular modeling to predict optimal structures *ab initio* that bind to FKBP but not effector molecules. Using the available X-ray crystal structure of FK-520 (or FK-506) bound to FKBP, molecular modeling can be used to predict polyketides that should optimally bind to FKBP but not calcineurin. Various macrolide structures can be generated by linking the ends of the FKBP-binding domain with "all possible" polyketide chains of variable length and substitution patterns that can be prepared by genetic manipulation of the FK-520 or FK-506 PKS gene cluster in accordance with the methods of the invention. The ground state conformations of the virtual library can be determined, and compounds that possess binding domains most likely to bind well to FKBP can be prepared and tested.

Once a compound is identified in accordance with the above approaches, the invention can be used to generate a focused library of analogs around the lead candidate, to "fine tune" the compound for optimal properties. Finally, the genetic engineering methods of the invention can be directed towards producing "chemical handles" that enable medicinal chemists to modify positions of the molecule previously inert to chemical modification. This opens the path to previously prohibited chemical optimization of lead compounds by time-proven approaches.

Moreover, the present invention provides polyketide compounds and the recombinant genes for the PKS enzymes that produce the compounds that have significant advantages over FK-506 and FK-520 and their analogs. The metabolism and pharmacokinetics of tacrolimus has been exstensively studied, and FK-520 is believed to be similar in these respects. Absorption of tacrolimus is rapid, variable, and incomplete from the gastrointestinal tract (Harrison's Principles of Internal Medicine, 14th edition, 1998, McGraw Hill, 14, 20, 21, 64-67). The mean bioavailability of the oral dosage form is 27%,

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trange 5 to 65%. The volume of distribution (VoID) based on plasma is 5 to 65 L per kg of body weight (L kg), and is much higher than the VoID based on whole blood concentrations, the difference reflecting the binding of tacrolimus to red blood cells. Whole blood concentrations may be 12 to 67 times the plasma concentrations. Protein binding is high (75 to 99%), primarily to albumin and alpha1-acid glycoprotein. The half-life for distribution is 0.9 hour; elimination is biphasic and variable: terminal-11.3 hr (range, 3.5 to 40.5 hours). The time to peak concentration is 0.5 to 4 hours after oral administration.

Tacrolimus is metabolized primarily by cytochrome P450 3A enzymes in the liver and small intestine. The drug is extensively metabolized with less than 1% excreted unchanged in urine. Because hepatic dysfunction decreases clearance of tacrolimus, doses have to be reduced substantially in primary graft non-function, especially in children. In addition, drugs that induce the cytochrome P450 3A enzymes reduce tacrolimus levels, while drugs that inhibit these P450s increase tacrolimus levels. Tacrolimus bioavailability doubles with co-administration of ketoconazole, a drug that inhibits P450 3A. See, Vincent et al., 1992, In vitro metabolism of FK-506 in rat, rabbit, and human liver microsomes: Identification of a major metabolite and of cytochrome P450 3A as the major enzymes responsible for its metabolism, Arch. Biochem. Biophys. 294: 454-460; Iwasaki et al., 1993. Isolation, identification, and biological activities of oxidative metabolites of FK-506, a potent immunosuppressive macrolide lactone, Drug Metabolism & Disposition 21: 971-977; Shiraga et al., 1994, Metabolism of FK-506, a potent immunosuppressive agent, by cytochrome P450 3A enzymes in rat, dog, and human liver microsomes, Biochem. Pharmacol. 47: 727-735; and Iwasaki et al., 1995. Further metabolism of FK-506 (Tacrolimus); Identification and biological activities of the metabolites oxidized at multiple sites of FK-506, Drug Metabolism & Disposition 23: 28-34. The cytochrome P450 3A subfamily of isozymes has been implicated as important in this degradative process.

Structures of the eight isolated metabolites formed by liver microsomes are shown in Figure 6. Four metabolites of FK-506 involve demethylation of the oxygens on carbons 13. 15, and 31, and hydroxylation of carbon 12. The 13-demethylated (hydroxy) compounds undergo cyclizations of the 13-hydroxy at C-10 to give MI, MVI and MVII, and the 12-hydroxy metabolite at C-10 to give I. Another four metabolites formed by oxidation of the four metabolites mentioned above were isolated by liver microsomes from dexamethasone treated rats. Three of these are metabolites doubly demethylated at the methoxy groups on carbons 15 and 31 (M-V), 13 and 31 (M-VI), and 13 and 15 (M-VII). The fourth, M-VIII,

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was the metabolite produced after demethylation of the 31-methoxy group, followed by formation of a fused ring system by further oxidation. Among the eight metabolites, M-II has immunosuppressive activity comparable to that of FK-500, whereas the other metabolites exhibit weak or negligible activities. Importantly, the major metabolite of human, dog, and rat liver microsomes is the 13-demethylated and cyclized FK-506 (M-I).

Thus, the major metabolism of FK-506 proceeds via 13-demethylation followed by cyclization to the inactive M-I, this representing about 90% of the metabolic products after a 10 minute incubation with liver microsomes. Analogs of tacrollimus that do not possess a C-13 methoxy group would not be susceptible to the first and most important 10 biotransformation in the destructive metabonsm of tacrolimus (i.e. cyclization of 13hydroxy to C-10). Thus, a 13-desmethoxy analog of FK-500 should have a longer half-life in the body than does FK-506. The C-13 methoxy group is believed not to be required for binding to FKBP or calcineurin. The C-13 methoxy is not present on the identical position of rapamycin, which binds to FKBP with equipotent affinity as tacrolimus. Also, analysis of 15 the 3-dimensional structure of the FKBP-tacrolimus-calcineurin complex shows that the C-13 methoxy has no interaction with FKBP and only a minor interaction with calcineurin. The present invention provides C-13-desmethoxy analogs of FK-506 and FK-520, as well as the recombinant genes that encode the PKS enzymes that catalyze their synthesis and host cells that produce the compounds.

These compounds exhibit, relative to their naturally occurring counterparts, prolonged immunosuppressive action *in vivo*, thereby allowing a lower dosage and or reduced frequency of administration. Dosing is more predictable, because the variability in FK-506 dosage is largely due to variation of metabolism rate. FK-506 levels in blood can vary widely depending on interactions with drugs that induce or inhibit cytochrome P450 3A (summarized in USP Drug Information for the Health Care Professional). Of particular importance are the numerous drugs that inhibit or compete for CYP 3A, because they increase FK-506 blood levels and lead to toxicity (Prograf package insert, Fujisawa DUS, Rev 4'97, Rec 6'97). Also important are the drugs that induce P450 3A (e.g. Dexamethasone), because they decrease FK-506 blood levels and reduce efficacy. Because the major site of CYP 3A action on FK-506 is removed in the analogs provided by the present invention, those analogs are not as susceptible to drug interactions as the naturally occurring compounds.

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Hyperglycemia, nephrotoxicity, and neurotoxicity are the most significant adverse effects resulting from the use of FK-500 and are believed to be similar for FK-520. Because these effects appear to occur primarily by the same mechanism as the immunosuppressive action (i.e. FKBP-calcineurin interaction), the intrinsic toxicity of the desmethoxy analogs may be similar to FK-506. However, toxicity of FK-506 is dose related and correlates with high blood levels of the drug (Prograf package insert, Fujisawa TUS, Rev 4-97, Rec 6/97). Because the levels of the compounds provided by the present invention should be more controllable, the incidence of toxicity should be significantly decreased with the 13-desmethox—nalogs. Some reports show that certain FK-506 metabolites are more toxic than FK-506 itself, and this provides an additional reason to expect that a CYP 3A resistant analog can have lower toxicity and a higher therapeutic index.

Thus, the present invention provides novel compounds related in structure to FK-506 and FK-520 but with improved properties. The invention also provides methods for making these compounds by fermentation of recombinant host cells, as well as the recombinant host cells, the recombinant vectors in those host cells, and the recombinant proteins encoded by those vectors. The present invention also provides other valuable materials useful in the construction of these recombinant vectors that have many other important applications as well. In particular, the present invention provides the FK-520 PKS genes, as well as certain genes involved in the biosynthesis of FK-520 in recombinant form.

FK-520 is produced at relatively low levels in the naturally occurring cells, *Streptomyces hygroscopicus* var. *ascomyceticus*, in which it was first identified. Thus, another benefit provided by the recombinant FK-520 PKS and related genes of the present invention is the ability to produce FK-520 in greater quantities in the recombinant host cells provided by the invention. The invention also provides methods for making novel FK-520 analogs, in addition to the desmethoxy analogs described above, and derivatives in recombinant host cells of any origin.

The biosynthesis of FK-520 involves the action of several enzymes. The FK-520 PKS enzyme, which is composed of the fkbA, fkbB, fkbC, and fkbP gene products, synthesizes the core structure of the molecule. There is also a hydroxylation at C-9 mediated by the P450 hydroxylase that is the fkbD gene product and that is oxidized by the fkbO gene product to result in the formation of a keto group at C-9. There is also a methylation at C-31 that is mediated by an O-methyltransferase that is the fkbM gene product. There are also methylations at the C-13 and C-15 positions by a methyltransferase believed to be encoded

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by the fkbG gene; this methyltransferase may act on the hydroxymalonyl CoA substrates prior to binding of the substrate to the AT domains of the PKS during polyketide synthesis. The present invention provides the genes encoding these enzymes in recombinant form. The invention also provides the genes encoding the enzymes involved in ethylmalonyl CoA and 2-hydroxymalonyl CoA biosynthesis in recombinant form. Moreover, the invention provides *Streptomyces hygroscopicus* var. *ascomyceucus* recombinant host cells lacking one or more of these genes that are useful in the production of useful compounds.

Moreover, if only one PKS gene is inactivated, the ability to produce FK-520 or an FK-520 derivative compound is restored by introduction of a recombinant expression vector that contains the functional gene in a modified or unmodified form. The introduced gene produces a gene product that, together with the other endogenous and functional gene products, produces the desired compound. This methodology enables one to produce FK-520 derivative compounds without requiring that all of the genes for the PKS enzyme be present on one or more expression vectors. Additional applications and benefits of such cells and methodology will be readily apparent to those of skill in the art after consideration of how the recombinant genes were isolated and employed in the construction of the compounds of the invention.

The FK-520 biosynthetic genes were isolated by the following procedure. Genomic DNA was isolated from *Streptomyces hygroscopicus* var. *ascomyceticus* (ATCC 14891) using the lysozyme/proteinase K protocol described in Genetic Manipulation of *Streptomyces* - A Laboratory Manual (Hopwood *et al.*, 1986). The average size of the DNA was estimated to be between 80 - 120 kb by electrophoresis on 0.3% agarose gels. A library was constructed in the SuperCosTM vector according to the manufacturer's instructions and with the reagents provided in the commercially available kit (Stratagene). Briefly, 100 µg of

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genomic DNA was partially digested with 4 units of SanSA1 for 20 min, in a reaction volume of 1 mil., and the fragments were dephosphorylated and ligated to SuperCos vector arms. The ligated DNA was packaged and used to infect log-stage XL1-BlueMR cells. A library of about 10,000 independent cosmid clones was obtained.

Based on recently published sequence from the FK-506 cluster (Motamedi and Shaffee, 1998, Eur. J. Biochem. 256: 528), a probe for the IEO gene was isolated from ATCC 14891 using PCR with degenerate primers. With this probe, a cosmid designated pKOS034-124 was isolated from the library. With probes made from the ends of cosmid pKOS034-124, an additional cosmid designated pKOS034-120 was isolated. These cosmids (pKOS034-124 and pKOS034-120) were shown to contain DNA inserts that overlap with one another. Initial sequence data from these two cosmids generated sequences similar to sequences from the FK-506 and rapamycin clusters, indicating that the inserts were from the FK-520 PKS gene cluster. Two EcoRI fragments were subcloned from cosmids pKOS034-124 and pKOS034-120. These subclones were used to prepare shotgun libraries by partial digestion with Sau3AI, gei purification of fragments between 1.5 kb and 3 kb in size, and ligation into the pLitmus28 vector (New England Biolabs). These libraries were sequenced using dye terminators on a Beckmann CEQ2000 capillary electrophoresis sequencer, according to the manufacturer's protocols.

To obtain cosmids containing sequence on the left and right sides of the sequenced region described above, a new cosmid library of ATCC 14891 DNA was prepared essentially as described above. This new library was screened with a new *fkbM* probe isolated using DNA from ATCC 14891. A probe representing the *fkbP* gene at the end of cosmid pKOS034-124 was also used. Several additional cosmids to the right of the previously sequenced region were identified. Cosmids pKOS065-C31 and pKOS065-C3 were identified and then mapped with restriction enzymes. Initial sequences from these cosmids were consistent with the expected organization of the cluster in this region. More extensive sequencing showed that both cosmids contained in addition to the desired sequences, other sequences not contiguous to the desired sequences on the host cell chromosomal DNA. Probing of additional cosmid libraries identified two additional cosmids, pKOS065-M27 and pKOS065-M21, that contained the desired sequences in a contiguous segment of chromosomal DNA. Cosmids pKOS034-124, pKOS034-120, pKOS065-M27, and pKOS065-M21 have been deposited with the American Type Culture Collection, Manassas, VA. USA. The complete nucleotide sequence of the coding

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sequences of the genes that encode the proteins of the FK-520 PKS are shown below but can also be determined from the cosmids of the invention deposited with the ATCC using standard methodology.

Referring to Figures 1 and 3, the FK-520 PKS gene cluster is composed of four open reading frames designated tkbB, fkbC, fkbA, and fkbP. The tkbB open reading frame encodes the loading module and the first four extender modules of the PKS. The tkbC open reading frame encodes extender modules five and six of the PKS. The tkbA open reading frame encodes extender modules seven, eight, nine, and ten of the PKS. The tkbP open reading frame encodes the NRPS of the PKS. Each of these genes can be isolated from the cosmids of the invention described above. The DNA sequences of these genes are provided below 10 preceded by the following table identifying the start and stop codons of the open reading frames of each gene and the modules and domains contained therein.

loading domain
domain g domain module 1 (KS1)
0

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KS2
     complement (37145 - 38296)
     complement (35749 - 37144)
                                       AT2
     complement (34606 - 35749)
                                       DH2 (inactive)
     complement (33$23 - 34480)
                                       KR2
     complement (33505 - 33715)
                                       ACP2
     complement (32185 - 33439)
                                       KS3
                                       AT3
     complement (31018 - 32185)
     complement (29869 - 31018)
                                       DH3 (inactive)
     complement (29092 - 29740)
                                       KR3
10
     complement (28750 - 28960)
                                       ACP3
     complement (27430 - 28684)
                                       KS4
     complement (26146 - 27430)
                                       AT4
     complement (24997 - 26146)
                                       DH4 (inactive)
     complement (24163 - 24373)
                                       ACP4
15
     complement (22653 - 23892)
                                       KS5
     complement (21420 - 22653)
                                       AT5
     complement (20241 - 21420)
                                       DH5
                                       KR5
     complement (19464 - 20097)
                                       ACP5
     complement (19116 - 19326)
20
                                       KS6
     complement (17820 - 19053)
                                       AT6
     complement (16587 - 17820)
                                       DH6
     complement (15438 - 16587)
                                       ER6
     complement (14517 - 15294)
                                       KR6
     complement (13761 - 14394)
                                       ACP6
25
     complement (13452 - 13662)
                                       KS7
     52362 - 53576
     53577 - 54716
                                       AT7
                                       DH7
     54717 - 55871
                                       ER7
     56019 - 56819
                                       KR7
30
     56943 - 57575
                                       ACP7
     57710 - 57920
                                       KS8
     57990 - 59243
                                       AT8
     59244 - 60398
                                      DH8 (inactive)
     60399 - 61412
                                       KR8
35
     61548 - 62180
                                       ACP8
     62328 - 62537
                                       KS9
     62598 - 63854
                                       AT9
     63855 - 65084
                                      DH9
     65085 - 66254
                                       ER9
40
     66399 - 67175
                                       KR9
     67299 - 67931
                                       ACP9
     68094 - 68303
                                       KS10
     68397 - 69653
                                       AT10
     69654 - 70985
                                       ACP10
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     71064 - 71273
         1 SATOTOAGGO AUGAAGTOOT COAGGOGAGG OGGOGAGGOO GUGAACACOT OGGOGGOTGOT
61 TGTACGGACO ACTIONGTOA JOGGOGATTO OGGAACGAG TOATGOGGAA TARAGGGGGG
        111 TTACAAGATO CTCACATTGO GOGACOGOCA GCATACGOTS AGTTGOCTCA GAGGCAAACO
        181 GAAAGGGCC GGGCGCTCCG CACCAGGGCG GAGTACGGGA CGAGAGTGGC GCACCCGCGC
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	2161	CGCCTGGACG	TGAGCGTAGT	TGCCGGCGGT	COAGCAGACG	GCCGTGGCAC	CGGTCGTCTG
	2221	CGCGGTGACC	GCGCCCGAGA	GCGGTCCGGC	CTTGCCGTCC	GCGTCCCGGG	CGGCGACCGC
35	1181	GTAGGTGTGC	GATGTGCCCG	CCCTCAGGCC	GGTGTCCGTG	TACGACGTCS	TGGCGGACGT
	2341	GGTGATCTGG	GCACCGTCGC	GGTGGACGGC	GTAGTCGGTG	GCGCCGTCGA	CGGGTTTCCA
	2401		ATGGTGGTGT	CGGTGGCGCC	3376603600	AGGCCGGACG	GAGCGGGGAG
		GGTGAGGGTG			GCCGAAGAAC		AGTAGCTGGA
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	1461	TGAACCACTS	CAMOTOCCGT	ATOTOCATGO	AGGGACTATA	COTACCOGGC	ATGGTCCTGG
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30	5183	GACCCCATGG	GAGGGACCCC		CGCATCCTCG		GAACGCCCCG
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	6061	TOCAGTOCGA	AGCGGGGCTG	GTCTCCATCA	CCGGCACCCC		TCCAAGGTGG
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	6961	TTCCCCGGGA	GCATGTTCCT	GGTGCTGGTC	GCCGTCACGT	TCCTCTTCGG	GATCGCCCGC
	7021	GTCAACGGCA	CGGTGGACTG	GCTGGTACGT	GTCGCGGTGC	GGGGGGGGG	GGCCCGGGTG
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30	9181	COGARGOGGT	CGCCCATGGT	GACGACGAGG	ACCTCATGGG	CGGGATCGGT	GAGCACGCGC
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	9301	GTTCCTCGAC	GCGGCTGAGT	TCCTCCTCCC	CCGCGGGGTGC	GATOGTCATG	GAGAGGTCGA
	9361	GOGAGOGOAG	GAAGTCCTCG	TCGGGACCGG	AGTACGCCTC	CCGGGGCCTGG	TCGCGCGCGA
	9421	AACCCGCCTG	GTACATCAGG	CGGCGCGGAC	GCGAGTCGAC	CCTGGACACC	GGCGGGCTGA
35	9481	ACTCCGGCAG	CGACAGGAGC	GTGGCCGCCT	GCTCGGCCGG	GTAGCACCGC	ACCTOGGGCA
	9541	GGTGGAACGC	CACCTOGGCA	CGCTCGGCGG	GCTGGTCGTC	GATGAACGCG	ATCGTGGTCG
	9601	STOCGAASTT	CAGCTCCGTG	GCGATCTCGC	GGACGGACTG	CSACTTCSSC	CCCCATCCGA
	9661	TGCGGGCCMG	CACGARGTAC	TOCGCORCAG	CGAGGCGTTC	CROROGOTICS	CACGCGAGGT
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	10561	ACCGCACCGG	ALCCETCTTC	CTGGAGACCG	AAGACGACCA	GGTGGTCCGC	GTAGGCGGCG
	10501	GCAGTCGTCC	LOBOTTOTO	GCCGTCG2CG	ACAGCGGTGT	CCCCGTCGAG	COGRACCOGC
	10001	GTOCGCATCG	COCECE CETC	COTECCCEC	TGCCGCTCAC	TGAAGCCGAC	GGCCGCGAGT
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		ACCCCCTGGA	3083003301	0300403443	20222222	CASCACCOCC	SATOGSCTTG
10		77000005000	000000000000000000000000000000000000000	3733333333	9099304708	227270700	SETCOEGGT
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15		PADSTGGGGG	70 21377	300000	3007093033	addadadaac	GAGTTGTGCC
`-	11341	GCCGCAGTT	CGGTGGCGAT	0000300030		SEATETOCTO	GGACGTGTCG
	12001	ACGAGTGTCA	CCGGGACGCC	GTGGCGCAGC	GCGAGCGTGG	TSATGCCGGT	GOCCATCACT
	12061	COCGCGCCGA	GCACGATCAG	CTGGTGGTCC	ACCOMMITTE	27772772233	GGTCACCATG
		GCAGCGAGTA	CGGGTCGAGG	ACGTCTTCCG	GGGTCGACGC	GATGGGGGG	TTGCGGCCGA
20	12181	GGCCGAGTTC	GTCGGCGAAG	CCGAGCAGCA	SOTEGAACGS	3/1737337723	GEGAACGCGC
20		TGCCGTCGA	GTOGAGGACG	CTCAGGCTGT	CCCGGTGGT	00000000000000000000000000000000000000	TCCGGTGCCG
	10301	030A0A6660	0300AGC0AC	GGGCCGAGCT	0333310333	02.31130130	TACTCGCCCT
	12321		oraccoccasA	TGGTCGACGG	AGATGAACGC	STOSTOSAGO	AGGTTTTCG
	12411	30%07770337	277322233	T06T033030	CGATGGCGTT	CACATOCAGO	TBCGGCAGCC
25	12481	0000000000	GEGEAGGACC	GGCCCTTTGC	CCGAGGGGAC		GTGGACAGGA
 .		3000000000	G3C3G3G3G3C	TOOGCOSSAT	CGGTCACCTT	GACCGGCAGT	CCGAGGAACG
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	10661	CGATGGGCAG		AGCGCGTGCG	COTGGGTCAI	coccrerece	COOGCGCCCGA
	15721	TOAGCGTGAG	CGTGGCGCTG	TOGGACCEGE	CCAGCAGCCG	GOTOGOGACG	GOGGCGACCG
30	10781			ATCACGCCTG	CGTCGGCGAG	GGCGGTCAGA	CTGCCGCTGT
517	12841		GCGCGACATC		mograsscas	CCBGAAGCGC	GGATAGTTGT
	12901	GOGGACTGTA		TTCATGGTCA	CGCCGACACC	GGGGACCCGG	TACGGCATGA
			GCCGGGAATG		GGACGAATCO	GGTACGCGGC	GGGGCCTCGG
				GCGGCGAACC	CGTCGTGCAG	CTOSCTGATO	AGCCGGTCCA
35	1182			ACGGAGAGAA	TCCGCTTGAT	GRICAGGTTGG	CGCAGGACCC
0.5	13141	TGGTCTGCAT		CCTTTCGTGG			CCGCTCGGGG
	13201	CGGCTTCCGT		GCTCCCTGTC	GATGAGGTOG		CCGCGGTCGC
	13261	STOCGOGGAC	AGCACGCCGG		cagadagara	1000000000	AGCGGTTGAG
	15.00	CAGGGGGGTCC	AGCCGGGTTC	CGATCGCGTC	CGCCTGGCGG	GOGCCCGGGT	CGACACCGGC
40	13361	AACGAGTGCT	TOCAGCOGGT		GAGGAGGAGG	GTCACCGGGT	CGTCCGGGGGA
	13441		CCGATGCSST	CGGCGAGTGC	GOGCGGGGAC	GGGTAGTCGA	AGADGAGCGT
	13501	GROGGECAST	CSCAGACCGG	TOGOCTOGTT	GAGGCCGTTG	CGCAGCTGCA	CCGCGATGAG
	1255	GGAGTCCACA	COGESTICCC	GGAACGCCGC	GTOCTOCGGG	ACSTCCTCCG	SSTCGGCGTG
	12500	202022222	GOOGGOOT	TOTGCCGGAC	GAGGGCGAGC	AGGTCGGTGG	GGCGTTCCTG
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	13811	CONTROL	ATGCCCTGTT	CGGCGGTGAG	CGCGCTCGCC	CCRCCCTTGC	GCATACGGCG
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50	13981	3772000300	GOGTAGTTGC	CCTGACCGGG	GGTGCCCAGC	ACACCGGCCG	CCGACGAGTA
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	14161	STOGAGGGTT	COGGCGGTGT	GGAAGACGGC	GGTGAGGGGT	TGAGGGATGT	GGGCGAGGGT
	14051	SSTGGGGAGT	#GGTGGGGGT	CGCCGACGTC	GCAGGGGAGG	TGGGTGCCGG	GGGTGGTGTC
55	14281	GGGGGGTGGG	GTGCGGGAGA	GGAGGTAGGT	GTGGGGGGTGG	TTCAGGTGGC	GGGCGAGGAT
	14341	GCCGGCGAGG	GTGCCGGAGC	CGCCGGTGAT	GACGACGGCC	CCCTCGGGGT	CCAGCGGCCG
	14497	cassaccers	AGGACGATCT	TGCCGGTGTG	CTCGCCGGGG	CTCATGGTCG	CCAGCGCCTC
	14461	SIGGRECTEC	CGCATGTCGT	GCACCGTCAC	CGGCAGCGGG	TGCAGCACAC	CGCGCGCGAA
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	15961	0700300703	GCGGCCTGCT	CGTCGGGGCAG	CGCCACCTCG	GCATACACGG	TGTTRCCATC
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35	16681	3000730000	CACGCCTGCG	CCAACGCCGT	CAGCCACCGC	TOCORGOGG	CGTCACCGST
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	16801	GCACTCCACG	AACACCGACC	CATCCAGCTC	CGCCACCGCC	GCGTCCAACG	CCACCGGACG
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50	17581	COCCECCO	accondacto	COGTOACOGS	GGCATCGCCC	AGCAGCACCG	CACGGTGACC
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	16061	GTTUGACTTC	AACGAACCCA	GCAGCAGCGG	AACCTCACGC	TOOTGOOGGT	AUGITUSUMS
	18121	AATGGCCTGC	GCCTCGATGG	GATCGCCCAG	CGTCGTCCCC	GTCCCGTGCG	CCTCCACCAC
60	13181	GTCCACATCG	GCGGGGGGGA	GTCCGGCGTT	CACCAACGCC	TGCTGGATGA	CACGCTGCTG

		1201333173	TT933333333	ACASCOCCTT	03833378023	TOOTSATTOA	CCGCCGACCC
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15	13071	ABBBARBBBT	300001 1030	CGACCAGAGC	2722.00/20	7772133333A	7660006006
	13141	137771131743	TOGRAGATAA	GCGTGGCGGG	CAUTIUGACA	177700000	COGCGAGTCS
		1010110130	tpGACGGCGG	TCAGCGAGTO	GATACCCAGT	TIPOTTORAGG	JOSCGTCCGC
		3383833777	4036067003	CGTGGCCGAG	GR00000300	3007737030	GGACCASTGO
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20	19381	3500070300	3003003330	GCGATACGGC	GOGGGGGAAAA	TODODGÁAAA	GOGGCGATGT
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			GTGCCCCTCA	1907900031	JAGTCCCCTG	T0%T0000000	AGAGGCCCCA
	19561	3370033773	AGCGCGGGCA	GTCGTTCGGC	ATGGCGCAGC	STOSCGASTO	CGTCGAGGAA
25	19621	330CA303A0		TGCCCTGGCC	3003000000	ADGATGCCCG	CGACGGACGA
25	19681	1000770300	GCCGAGTAGT			10073CAGGT	GCCAGGCGCC
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30	19981	COCCEGGCGGT	TOGOTGOGOG	ACAGCAACAG	GAGGTGGCGG	GCGCCATGCT	CGGCGACGAG
	20041	AMGCDGGGGG	AGGAGACCTG	CCAGCACACT	CGAGCCGCCG	GTGATGACCA	COGTGCCGTC
	20131	CGGGTCGAGC	ASCSSTTC3G	GCGTTTCCGC	GGCGGCCGTG	COSCTSARCO	GOGGGGGTTC
	26161	GTACCGGCCG	TOSGTGACGC	GGACGTACCG	CTCGGCCAGT	3733733663	OGGCCAGCGC
	20221	CTCOMTGGGG	3737333730	CGGTCTCCAC	CAGCACGAAC	COGCCCGGGT	GCTCGGCCTG
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	20341	3833313310	TCCGCAGGGC	CGTCCTCGGC	GATCACCCGG	TGCAGCTCGC	CGAGCACGAA
	20401	CTCGGTGAGG	COGRACGIOT	CGTCGAGGAC	ATCCGCGCCCC	GBTTTCGGGGA	GCGCGGAGAC
	20461	SATGT3SACC	GOGTOCGOAG	GACCGGGCCC	GGGAGTGGGC	ABSTRAGTCS	AGGAGAGGCC
	10501	GIRCHAGGAG	TTOCOTACGA	CGGCGGCGTC	GCCGTCGACG	TTORUGGGTC	GCGCGGTCAG
40	10589	73733333A	STCACCAGOG	GTTGGCCGAC	casatteeste	GOATGUACGG	CAGOSCOSTO
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	22831	DACK JOAGO	GTCTTCGGCG	CGATGCCATA	COCCATOCCC	ATGROGATOT	TGATGACACC
	213861	GGGGACACCC	GCAGCGGCCT	GCGCATGACC	GATGTTCGAC	TTCARCGRAC	CCAGCAGCAG
	22921			CGTACGTCSC			
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	23041			TGACGCGCTG		COSTTOGGTG	
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	23161	GIRGGGGGGG	POPOSOS SE	GCTTTTCGAC			
2.5	23221			CGAACGCCTT		TODGGGGGGA	
25				TOTGTGGTGA		GTGACACCAC	
	23341			GCAGCGCCTG			
				TGACCGCCGG			
	23461	TCCGGCGAGC	ACCGCGGGGCT	GTGTGCTGTA	GGCGCCGAAT	CCGCCCAGGT	
	23521			CGCCGACGAA		TOGGTGCCGC	GCAGGGTGTC
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	23641	CGGGTCGAGT	GCGGTGGCCT	CGCGCGGACT	GATGCCGAAG	AACGCGGCAT	CGAAGTCGGC
	23701	GGGGGGGGG	AGTGCGCCGG	CCCGCCCGGT	GGCGGACTCG	GOGGGGGGGT	GCAGCGCGGC
	23761			TGGGGAAGTC		TOSOGGCCGT	
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17)	24121			ACGTCCGGGG		CAGCAGATGA	
40	14181	CGCCGGCGGC	GGGATAGTCG	AAGACGAGCG	166006665	OGGAATGCCG	
	24241	AGAGCCGGTT	GCGCAGGCCG	AGCGCGGTGA	GCGAG'TCGAC	COCGAGGTCC	TTGAACGCCG
	24301	TAGIGGCCGT	GACCGCCGCC	GCGTCGGTGT	GGCCCAGCAG	GGTGGCGGCG	GTGTCGCGGA
	24361	CGACGCCGAG	CAGCACCTGT	TCCCGTTCCT	TSTGGGGCAG	STCCGGCAGG	CGTTCCAGCA
	24421	GGGAGCCGCC	GTCGGTCGCG	GAGCGCCGGG	TGGGGCGCTG	GATCGGTCGC	CACAGCGGTG
45	24481	ACGGGTCGCC	SGGCCCGGGT	GGGGCGGTCS	CCACGACCAC	GGCTTCCCCG	GTGGCGCACG
	24541	CGGCGTCGAG	GAGGTCGGTC	AGCCGGTCCG	CCGCGGCGGT	GAACGCCACG	GCCGGCAGGC
	24601	CTTGTGCCCG	SCGCAGGTCG	GCCAGGGCCT	GGAGCGGTCC	GÉCCGCCTCG	CCGGACGGAA
	24661	CGGCGAGAAC	GAACGCGGTC	AGGTCGAGGT	CGCGGGTCAG	GOGGTGCAGT	TOCCAGGCCG
	2,201	ECTOCOCCC	30037000010	TGGACGACCG	CGGTCACCGG	GGTTTCCGGC	ACTGTGCCCG
50	1701	ACTC33C331	3000100000	GCGCCGTGTC	00000000000	THERECORDE	TCCTCCG3AC
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55	15081	ROACCACCAG	CGTGGCGCCG	GCGGTCCTCG	GSTCGTCCAS	1.0000TACGG	ACCTOGTOGG
	25141	JACCGGATAC	CGGGACGACG	ATGACGTCGG	GCGTGGCGTC	STCSCCGAGG	TCGGTGTACC
	25201	SGCGGGCCGT	GGTGCCGGGT	GCCGCCGGGG	CCCGGACGCT	SGTCCAGGTG	CGCCGGAACA
	15061	GCCGCACGTC	COOGTOCGGG	CCCGTCGTGG	CGGGGGGGCCG	GGTGATGAGC	GAGCCGATCT
	25321	GAGCCACCGG	COGTOCCAGT	TOGTOGGOGA	GGTGCACGCG	GGCGCCGCCC	TEGECETEGE
60	25381	CGTGGACGAR	GGTGACGCGC	AGTTTCGTGG	CGCCGCTGGT	GTGGACACGG	ACGCCGGTGA

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	27061	SCATOCTEGG		GGGGAGGCCG	CCATCAGTTO	SACGUCCATG	CCGCGCCACT
	27171	GCGGTCCTTG	TCCGGGGAAG		TGCGCGGCTC	SOTGRECO	STOCCGGTGA
30	171,81	CGACGTCGTC		ACGGCGCGGT	GCGGGAACGT	COTACGCCTG	GCGAGCAGGC
	27341	CCGCGGCGAT	GGCGCGCGGG			GAGGTGCTCG	CGGAGTCGGC
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50	20.25	GOGOCFOCA	CGAGGTCTCC	AGGECCAGAC	SCTGCTGCGG	GTCCATCGCC	AGOGCCTCAC
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30	30781	TGARCGTGTC	GAGGGGGGGG	CAGCCGGCTT	CONTOGERED	CCGGATCGCC	TOGGCGGCGT
	30841	GGGCCGCGGC	GGGCAGCACC	GCGAGGCCGT	CTERECOGACC		GOGGTCAGCG
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25	34081	ACOTGGGGGA	TATGGACGAG	TACAGGACGA	ACGCGGCCAG	STOSAGATOS	CGCGTCAGCT
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40	34981	GGACCGGTGA	SCCGTGCTCG	TOCGTGGCGA	CGATGCGGAC	0ATGT06333	CCGACGCGTT
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•	5 55 4 4 4	3043049003	SATGTCSCCG	TCCCACTCGA	131333333		ADGTGCAGOG
							ACCIDENCES OF
		T0100000A3	GACGCCGTGC	ACCCCTADEC	TSACCATOT:	in Drugged da	SDDDBCASSS
	37161	2000030000	GGTGTGGCCS	ATGTTOGACT	TGAGJGAG0 '	MAT DAGGAGD	GBATGCACGC
	37321	:TT20003001	3710300400	TGCAGGGCCT	GGGCCTCGAI	3033703000	AGACGGGTGC
20		000700073		GOGTCGACGT	CACCOGGCGC		TOGGOGAGOG
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	37441	CRESCRESAT	GROGOGOTGO	TGCGCAGGCC	93 TT 0 33 033		TTCSACGCGC
	37531	CGTCGGAGTT	GACCGCGGAG	CCGCGCACAA	GCGCCAGCAC	70000000000000000000000000000000000000	TBBCGGGTGG
	37561			ACCAGGAGAG	0330300073	GGGGAAGCTC	STBCCGTCCG
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25	37681	DBACGAACCC	GGTCGTCGTC	GCCATCACCG	. W.A.CHOUSSE	MOCAGGGGG	
	37741	COCCCGAGCG	CAGCGACCGC	GCGGCCTGGT	GCAGCGCCAC		GAACACGCCG
	37801	TGTCGACGGT	BACCGACGGG	CCCTCCAGAC	CGAAGTAGTA	COAGAGCCGC	CCGGAGAGAA
				GCCCCGAFAC	CGCCCAGGTC	CHOSCOCOS	
	37861	COCTAGTEGG					
	37921	GGGTGRACGC	SCCCATGAAT	ACGCCGGTGT	CGCTGCCGCG	GACGOTTTCG	
30	37981	COGCTCGTTC	SAACGCCTCC	CACGACGCTT	CGAGGACCAG	ADSCIGCTGC	GGGTCCATCG
	38041			ATCCCGAAGA	ACGCGGCGTC	SAAGTOGGOG	GCGCCGGTGA
				GAAACCTTGC	CGACCGCGTC	GGGGTTCGGG	TOGTAGAGOG
	38101		GTGACGCACG				
	38161	CGGCGAGGTC	CCAGCCGCGG	TOGGOGGGGA	ACTOGGTGAT	COCCTCCCC	
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35	38291	CGATOSCOAS		CCCGCCACCG	TOSGTGC333	TRITIGIOGIC	GOOGGAGCGG
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	35641	GCTCGGTCCG	CTGCCGGACG	GGCTCGGTGG	GAATCGCCGC	GACCATGAAC	
	38701	CGGCGAGGCT	CGCGTCGATG	AAGTGGGTGC	CCTCGGCCTC	GETGAGCGGC	JGGAACCCG'L
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ر	41251	COTOCAGGCT	CGGCACGACC	COATGCGCCT	gggAcAg0gc	SSUCAGGCTS	ACCGCGACCG
	41341	CORCAGOSTO		ACCTCCACCC	GCTCCGCCAC	ATCCGACCGC	GACAACATCT
	41401	CCCACCTGGC	CGGCTGGACC		ADGCCCGCGC	ACACTCCTCC	ATACGAGCCG
	41461	CCCGCACATO	COAGCCCGTG	TGCGGCAACA	CGCCCATGCC	CACCCACTGG	GCACCCTGCC
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	42601	TETOGREEST	GACCGCCGGG	CCCTGAAGTC	CGTACACGTA	CGAGAGGCGU	CUGGACAGGA
	42661	DGCTCGTCTG	CGTCGCCGTG	ACACCGAGCC	CGCCCAGGTC	CCGGCCGACG	CCGTAGCCCT
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         45001 GCCGCTCCCA GACCASTTCG CACAGCGTGS CCTCGCCACT 30033TGGCG ACCAGATGGS
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45081 CCGGCAGOOC CGCGAGOGC GCGCGCTGGA COTTGCCCGA TSCGGTGGG GGGATCSTGG
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          45241 CGAGGACGGG GTGCGGGCGG CCCGCCGCGG CGGCGTCCCG GACACCGGCC ACCTCCTGGG
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          45541 CCGGGTCGAC GAACCGCAGC GACAGGCCCG GCACGGGCAG CCCGCAACGAG CCGGGAAACCC
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          45661 CGAGCAGGGG CACGCCGAAC GTCGCCTCGA AATCCCTGGT
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          AETAI ATTOGGOGAT CAGOSCOACG OGCAGTGOGS CAGCCCGGGG CTTGGCGGGAC ACGGCGCGA
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         45981 STTOSTOSTO STOSSTORGO CGCCAGGACG GCACGTCGCA STSCATCGCG GACCACAGGC
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          46141 CGAGGTCCTC GTAGGAGACG CAGTCCGGTG CCCGGCGCCC GAGGAGCACG ACGGTGGCGT
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          46261 CGGAGTCCGT CAGGAAGTGG GCGAGTTCGG CGTCGGCGGC GTCCGGGTTG AGCGGGACGG
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          46441 GGCCGCCCCG GAGCCGGAGT TGCGTGTACG TCACGGCGCG TTGGGAATCC GTGTAGGCGA
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	48601	TTCATGACCC	TOCTOGGGGG	CTTCCAAGGG	GTCCTCGCCC	GGCAGGCGGG	CACGCGGGAC
		STGCTGGTCG	700700000	#2004Z0020	ACGCGGGGGGG	CSTACGAGGG	CCTGATCGGC
	48661	ATGTTCGTCA	GCACGCCCG.	GOCOMACCOI	Cr CCTCTCTCCC	GCGATCCGTC	CTTCCCGGGAA
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	48841	GAGAACGTCA	TOGAROTOGT	CGCACCGGAA	CGCGACCTGT	UGG_UAACCU	OBLUGITUMO
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45	19681	TOOGGGGGGGG	innegenagene	GAAGGCCGTG	CTCATGCCGG	GTGTCAGCGC	CGTCAACCTG
	49741	CTGCTCTGGG	AGGROCAGE	GATGGGCAGG	GARCCGGCCA	GCCGCACCGT	CCAGTTCGTG
	19901	ACCCCACGT	magachacho	GGTGCAGGAG	ATCTTTTCCG	CGCTGCTGGG	CGGCACGCTC
	40061	GTGATGGGGG	CCCNCCNCCT	GCGGTTGGEC	CCGCCGGGGAC	TOGCOCGGTG	GATGGACGAA
	49501	CASCOGATTA	222222222	00001100110	CCCCTTCTGC	CTCTCCTCET	CGRGCACGTC
- 0	49921	Cabologaira	CCCGGWTCTW	CGCGCCGACG	0000100737	63636666	CONCOCOMO
50	19961	GATCCSCACA	GCGACCAGUT	Caccata	CGGCACCIGI	000000000000000000000000000000000000000	CGMCCCCCCC
	50041	ATCCTCGACG	CGCGGTTGCG	CGAGCTGTGC	CGGCACCGGC	فالماديات	المصالحات والا
	50101	CACTACGGTC	CGGCCGAAAG	CCAGCTCATC	ACCGGGTACA	CGCTGCCGC	CGACCCCGAC
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	4,000	GACGAGGCGA	mgngggaaaa	TOCGGACGST	ATGCCGGGGC	AGCTOTGCGT	COCCGGCGTC
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	52621	AGGCGTTCGA	GCACGCGGGC	ATCGATOCGC		GGGCAGTGAC	
35	52631	TOCTOGGCGC	GTTCTTCCAG	GGGTAGGGCA	TCGGGGGGGA	CITTEGACGGT	TACGGCACCA
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50	53581	ACCCCCGACC	GGCCCCCGAA	CCCGCCCCCG	CACCCGACAC	CGGACCGCTG	codoraciac
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25	9147		COCCUTOCGOG	GACGATOCSC		Cat coreage	GACCCCCCC
25	13451	ACGCCGACGT	TCCGGCGGGC	ACCCGGGAGG	TOACOGCOCG		GCSCTCCAGC
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30	55981	ACCCCGCGCA	CGGCCCGCTG	TCCCTGCCGG	ACGGCGACTG	GCTGCTCACC	CGGTCCGCCT
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	56101	CCGGCGAGGT	CCGCATCGAC	GTCCGCGCGG	CCGGACTGAA	CTTCCGCGAT	GTGCTGATCG
	56161	COCTOGGGAC	GTACACCGGG	GCCACGGCCA	TGGGCGGCGA	GGCCGCGGGC	GTCGTGGTGG
	86251	AGACCGGGCC	CGGCGTGGAC	GACCTGTCCC	COGGCGACCG	GGTGTTCGGC	CTGACCCGGG
35	5.6281	GOGGCATCGG		GTCACCGACC	GGGGGGGGT	GGCCCGGATC	CCCGACGGCT
-	56541	GGAGCTTCAC		TCCGTCCCGA	TOSTGTTOGC	GACCGCGTGG	TACGGCCTGG
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	57061	GGACGCCCGG	CGTCCACCTG	CCCTGCGACG	TOGGTGACCG	GGACCAGCTG	GCGGCGGCCCC
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50	57191	SCACCGTCGC	GTCGCTCACC	CCCGAGCGTT	TOGACACGGT	GCTGCGCCCG	AAGGCCGACG
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	59521	AGCATCCCGC	GATGGGCGAG	CAGCTCGCCG	CCGCCCATCC	CGTGTTCGCC	GACGCCTGGC
30	59581	ATGAAGCGCT	CCGCCGCCTT	GACAACCCCG	ACCCCCACGA	COCCACGCAC	AGCCAGCATG
	59641	TGCTCTTCGC	CCACCAGGCG	GCGTTCACCG	CCCTCCTGCG	GTCCTGGGGC	ATCACCCCCC
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35	59891	CGGGCGTGGA	GATCGCCGCC	GTCAACGGGC	CUCECIOCE		
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	60001	CCGGGCACTC	CGCGCACATG	GAGCCCGTGG	CCGCCGAGCT	SCTCSCCACC	
	60061	TOOGCTACCA	CCCTCCCCAC	ACCTCCATTC	CGAACGACDC	CACCACCGCT	
		CCGAGGAGGT	COSCRAGOCO	GTGGTGTTCC	ACGCCCACGC	GCAGCAGTAC	CCGGACGCCG
40	80181	TGTTCGTGGA		GCCCAGGACC	TCTCCCCGCT	COTOGROGGG	ATCCCGCTGC
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	an To 1	CCGAGGGGGT	SCTSCSCCC	CATGGCACGG	CCCTGCCGGA	TGCGGCCGAC	GCCGAGTGGC
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50	00701	TCTTCGCCGA	222212222	CTCCCTCCCC	P.C.C.TTTCCT	CONTRACTOR	SACCTGCTCS
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55	21081	CGCTGCGGGA	GGTGGCGTCA	CCGTCCGGCT	CCGAGGAGTC	GGACGGCCTS	CACCGGTTGG
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10	61961	SCCASSCASS	GCACACCCTC	9600AA0000			ATGTGGCACA
	61041	00A00AG0A0	CCTCACCGGA	=	ACGCCGACCG	SGACCSCATC	CGCCGCGGCG
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	62401	CGACGTTCAA		ATCGACTCGC	TCACCGCGAT	CGAGCTGCGC	AACCGGCTCG
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	aces s		GACCTACGTC	CGGCACGGCG	3077007030	deasecosco	GGCTTCGATG
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<u> </u>	62881			744000000000	TOTOCCATGG		GGCGTCGACC
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	54501	CCGGTGAGGT	CGGTCTGGTC	TOCCOCCETO!	* CONCGROOM	GLOGOGOTAT	GAGACCGAAG
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                          ACCIACON COTACCTECO COACGETETO GAZCETETO JEGAGGACET GETOTECACO COACGETETO GAZCETETO ACACCECATO COTOCCOCATO CONOCCOCATO CONOCCO
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             75241 GGGTGCGGGA ACCGCCCTTC CCGCAGCAGC CGCCCCTCGA CCAGCTGTTC GTGGGCCTGC
             78301 TOGACOGOT OGGTGTOGAG GCOGGTCATO CGCTGGACGA GGGTGAGTTC GACACTCTOG
             78381 COGAGOACG CGGAAGCTCG GGCGACGCTC AGCGCGGCGG GGCCGCAACG ATAGAGCGAC
             75421 CCGAGGTAGG CGAGCCGGTA CGCCGGCCCC GCGACCACTT CCAGGGACCC TGAGGTCCGT
             75481 GTCCGTCCCT COCGGATGTC GTCGATCAGG CCGTGGCCGA GGAGCAGGTT GCCGCGGGCC
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             78541 GCCCGGAACG CCTGGGCCAC CACGTCGTCG TGCGCGTCCT GGCCGAGGTG CCGGCGCACG
              75691 AGTTOGGTGG TOTGOGCOTO GGTGAGOGGG OGCAGOGGGA TOTCOTGGTA GTGGCGOAGA
             75881 CTCAGCACTE CCGCCCGGAA TTGGGAGTGG GCGGGCGTCG GCCGGAGCAG CTCGGTCAGC
             75721 ACGATGGCGA CACGGGCCCG GCTGATGCGG CGCGCGAGGT GGAGCAGGCA GCGCAGCGAC
             75781 GGCGCGTCGG CGTGGTGCAC GTCGTCGATG CCGATCAGTA CGGGCCGCTC CGCGGCGAGC
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TERAT STOAGLANDS TECRETORS THOUSTOND ASSOCIATED PRACETORS CASCAGETT TERMS TOODACKATE COSTRACOO SACCASCIDE SITETICESS DESCOAGETO ASSOTIGETO TBAKI AGGAGITGSI CGAGCATGCO GTACGGCASA GCCCGCTCCT CCATGGAGCA CACCGCGCGA WILL AGGGTGACCA AGGCGGCCTT GGCCGCGGGG GCCTUGAGGA GTCGGGGCCTT GCCGCAGGCG TAREST AT DESCRIPTION TRANSPORTED SACRACERS CONTROL SUBCTORES GASCACCOS TRIBLE TOURS GRANT CHARTESTS ATCRESSES ATCRESTOT GUGGATAR GOSCECTATO TRIBLE ACGRATGERA CTRESTESS ACCOTTOSTES ARRECTATO SCRIPTARE SCTTETTER MADAL OTOPACOGOT GUGATTOAGO OTGGGGGGAT GOTGTGOTAT AGATGGGAAG ATGTGATOTA MADAL GGGGGGTGCG OTTGGGTGAG GAGGGGACCG CTCCGGGGGGGCG AACCGGGGGG -2321 GGGCCGTCCC GTTCCCTCAG GAGCCGALTG CICCCGGJE, WALCCGCCG ACCCCCCAGGGAAGA 18381 CCACCAGCTC GGCGACCGCC TCCTGGTGGT CGACGAGGTA WAAGTGCCG CCGGGGAAGA 10 NASE CONCORDED A MACCOCCO GEOGRAPHIC CONCORDED AND CONCORDED CONCO 15 TABLE GUAGOTOGRE STOLLANGES AGGEGETES SELEGIOSES CAGOTOGRAD GUAGOCOGO COMPLAT GUAGOTOGRAD GATCANGTGO GOCACOGGGA GUAGOTOGRAD CAGOCOTOGRAD GUAGOCOTOGRAD GUAGO 20 TERRI GOTAGAADET DECOGATOOG COGGOTTGG GOAGCAGDAD DADDCOTAGO GGGGOOTTGG GOAGCAGDAD DADDCOTAGO GGGGOOTTGG GOAGCTGAD DECAGCOCCT CGGCGGGAC TRIBLE OTGSGGAGOS COSTACOGGG TGATCTOSSC CHAGTGCTTS TOTGGGATCT COSGSTAGGT 77181 CACGCCCAT CONTICCTOOG GOGCCAGACA GAGGACGCC ACTTTGCCGT TGTGCACATT 77221 GOGATGCACA TOGOGCACCO COGACCOGAC GTOGOCTC MATTIGOGIA COGACAGCGT 77221 GOGATGCACA TOGOGCTTGC AGATCAGGCG GTTCGCCTTC CACGCCTCAC GATAGTTCGC 77341 GAAGTGGGTA COGATGATCC GCTTCACGGA CATCCACAGG TACCGATTGT CAAAGGCGTG 25 T7401 CTCGTATCC2 GAGGTTGACG CGCAGGTGAC GATCGTGCCA CCCCGACGTG TCACGTAGAC DOMEST ACTOGOGOGG ARCOTOGOGG GCCCCGGGGG CTCGARCAGS ATRICGGGGAT CGTCRCCCCC TIB21 GGTCAGCTCC CGGATC 30

Those of skill in the art will recognize that, due to the degenerate nature of the genetic code, a variety of DNA compounds differing in their nucleotide sequences can be used to encode a given amino acid sequence of the invention. The native DNA sequence encoding the FK-520 PKS of *Streptomyces hygroscopicus* is shown herein merely to illustrate a preferred embodiment of the invention, and the present invention includes DNA compounds of any sequence that encode the amino acid sequences of the polypeptides and proteins of the invention. In similar fashion, a polype ide can typically tolerate one or more amino acid substitutions, deletions, and insertions in its amino acid sequence without loss or significant loss of a desired activity. The present invention includes such polypeptides with alternate amino acid sequences, and the amino acid sequences shown merely illustrate preferred embodiments of the invention.

The recombinant nucleic acids, proteins, and peptides of the invention are many and diverse. To facilitate an understanding of the invention and the diverse compounds and methods provided thereby, the following general description of the FK-520 PKS genes and modules of the PKS proteins encoded thereby is provided. This general description is followed by a more detailed description of the various domains and modules of the FK-520

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PKS contained in and encoded by the compounds of the invention. In this description, reference to a heterologous PKS refers to any PKS other than the FK-520 PKS. Unless otherwise indicated, reference to a PKS includes reference to a portion of a PKS. Moreover, reference to a domain, module, or PKS includes reference to the nucleic acids encoding the same and vice-versa, because the methods and reagents of the invention provide or enable one to prepare proteins and the nucleic acids that encode them.

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The FK-520 PKS is composed of three proteins encoded by three genes designated fkb.4. fkbB, and fkbC. The fkb.4 ORF encodes extender modules 7 - 10 of the PKS. The fkbB ORF encodes the loading module (the CoA ligase) and extender modules 1 - 4 of the PKS. The fkbC ORF encodes extender modules 5 - 6 of the PKS. The fkbP ORF encodes the NRPS that attaches the pipecolic acid and cyclizes the FK-520 polyketide.

The loading module of the FK-520 PKS includes a CoA ligase, an ER domain, and an ACP domain. The starter building block or unit for FK-520 is believed to be a dihydroxycyclohexene carboxylic acid, which is derived from shikimate. The recombinant DNA compounds of the invention that encode the loading module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of methods and in a variety of compounds. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 loading module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for the loading module of the heterologous PKS is replaced by the coding sequence for the FK-520 loading module, provides a novel PKS coding sequence. Examples of heterologous PKS coding sequences include the rapamycin. FK-506, rifamycin, and avermectin PKS coding sequences. In another embodiment, a DNA compound comprising a sequence that encodes the FK-520 loading module is inserted into a DNA compound that comprises the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the loading module coding sequence is utilized in conjunction with a heterologous coding sequence. In this embodiment, the invention provides, for example, either replacing the CoA ligase with a different CoA ligase, deleting the ER, or replacing the ER with a different ER. In addition, or alternatively, the ACP can be replaced by another ACP. In similar fashion, the corresponding domains in another loading or extender module can be replaced by one or more domains of the FK-520 PKS. The resulting heterologous loading module coding sequence can be utilized in conjunction

with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide.

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The first extender module of the FK-520 PKS includes a KS domain, an AT domain specific for methylmalonyl CoA, a DH domain, a KR domain, and an ACP domain. The recombinant DNA compounds of the invention that encode the first extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 first extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence a module of the heterologous PKS is either replaced by that for the first extender module of the FK-520 PKS or the latter is merely added to coding sequences for modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the first extender module of the FK-520 PKS is inserted into a DNA compound that comprises the remainder of the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, all or only a portion of the first extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2hydroxymalonyl CoA specific AT; deleting either the DH or KR or both; replacing the DH or KR or both with another DH or KR; and/or inserting an ER. In replacing or inserting KR, DH, and ER domains, it is often beneficial to replace the existing KR, DH, and ER domains with the complete set of domains desired from another module. Thus, if one desires to insert an ER domain, one may simply replace the existing KR and DH domains with a KR, DH, and ER set of domains from a module containing such domains. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a gene for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous first extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous

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PKS can be replaced by one or more domains of the first extender module of the FK-520 PKS.

In an iliustrative embodiment of this aspect of the invention, the invention provides recombinant PKSs and recombinant DNA compounds and vectors that encode such PKSs in which the KS domain of the first extender module has been inactivated. Such constructs are especially useful when placed in translational reading frame with the remaining modules and domains of an FK-520 or FK-520 derivative PKS. The utility of these constructs is that host cells expressing, or cell free extracts containing, the PKS encoded thereby can be fed or supplied with N-acylevsteami — thioesters of novel precursor molecules to prepare FK-520 derivatives. See U.S. patent application Serial No. 60/117,384, filed 27 Jan. 1999, and PCT patent publication Nos. US97/02358 and US99/03986, each of which is incorporated herein by reference.

The second extender module of the FK-520 PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, an inactive DH, and an ACP. The recombinant DNA compounds of the invention that encode the second extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 second extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the second extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the second extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, all or a portion of the second extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the KR and/or the inactive DH; replacing the KR with another KR; and/or inserting an active DH or an active DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these

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replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous second extender module coding sequence can be utilized in conjunction with a coding sequence from a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the second extender module of the FK-520 PKS.

The third extender module of the FK-520 PKS includes a KS, an AT specific for malonyl CoA, a KR, an inactive DH, and an ACP. The recombinant DNA compounds of the invention that encode the third extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 third extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the third extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the third extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, all or a portion of the third extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting the Kr. and/or the inactive DH; replacing the KR with another KR; and/or inserting an active DH or an active DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous third extender module coding sequence can be utilized in conjunction with a coding sequence from a PKS that synthesizes FK-520, an

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FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the third extender module of the FK-520 PKS.

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The fourth extender module of the FK-520 PKS includes a KS, an AT that binds 5 ethylmalonyl CoA, an inactive DH, and an ACP. The recombinant DNA compounds of the invention that encode the fourth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 fourth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS 10 is either replaced by that for the fourth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the fourth extender module of the FK-520 PKS is inserted into a 15 DNA compound that comprises the remainder of the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the fourth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the ethylmalonyl CoA specific AT with a malonyl CoA, methylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; and/or deleting the inactive DH, inserting a KR, a KR and an active DH, or a KR, an active DH, and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, a PKS for a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous fourth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the fourth extender module of the FK-520 PKS.

As illustrative examples, the present invention provides recombinant genes, vectors, and host cells that result from the conversion of the FK-506 PKS to an FK-520 PKS and vice-versa. In one embodiment, the invention provides a recombinant set of FK-506 PKS

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genes but in which the coding sequences for the fourth extender module or at least those for the AT domain in the fourth extender module of the FK-520 PKS. This recombinant PKS can be used to produce FK-520 in recombinant host cells. In another embodiment, the invention provides a recombinant set of FK-520 PKS genes but in which the coding sequences for the fourth extender module or at least those for the AT domain in the fourth extender module have been replaced by those for the AT domain of the fourth extender module of the FK-506 PKS. This recombinant PKS can be used to produce FK-506 in recombinant host cells.

Other examples of hybrid PKS enzyman of the invention include those in which the AT domain of module 4 has been replaced with a malonyl specific AT domain to provide a PKS that produces 21-desethyl-FK520 or with a methylmalonyl specific AT domain to provide a PKS that produces 21-desethyl-21-methyl-FK520. Another hybrid PKS of the invention is prepared by replacing the AT and inactive KR domain of FK-520 extender module 4 with a methylmalonyl specific AT and an active KR domain, such as, for example, from module 2 of the DEBS or oleandolide PKS enzymes, to produce 21-desethyl-21-methyl-22-desoxo-22-hydroxy-FK520. The compounds produced by these hybrid PKS enzymes are neurotrophins.

The fifth extender module of the FK-520 PKS includes a KS, an AT that binds methylmalonyl CoA, a DH, a KR, and an ACP. The recombinant DNA compounds of the invention that encode the fifth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 fifth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the fifth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS. In another embodiment, a DNA compound comprising a sequence that encodes the fifth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the fifth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA

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specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one or both of the DH and KR; replacing any one or both of the DH and KR with either a KR and/or DH; and/or inserting an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous fifth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes $vK = \sin FK$ -520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the fifth extender module of the FK-520 PKS.

In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the DH domain of the fifth extender module have been deleted or mutated to render the DH non-functional. In one such mutated gene, the KR and DH coding sequences are replaced with those encoding only a KR domain from another PKS gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that lacks the C-19 to C-20 double bond of FK-520 and has a C-20 hydroxyl group. Such analogs are preferred neurotrophins, because they have little or no immunosuppressant activity. This recombinant fifth extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this fifth extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (lacking the C-19 to C-20 double bond of FK-506 and having a C-20 hydroxyl group) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the DH domain of module 5 has been deleted or otherwise rendered inactive and thus produces this novel polyketide.

The sixth extender module of the FK-520 PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, a DH, an ER, and an ACP. The recombinant DNA compounds

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of the invention that encode the sixth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 sixth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the sixth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the sixth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

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In another embodiment, a portion of the sixth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one, two, or all three of the KR, DH, and ER; and/or replacing any one, two, or all three of the KR, DH, and ER with another KR, DH, and ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous sixth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the sixth extender module of the FK-520 PKS.

In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the DH and ER domains of the sixth extender module have been deleted or mutated to render them non-functional. In one such mutated gene, the KR, ER, and DH coding sequences are replaced with those encoding only a KR domain from another PKS gene. This can also be accomplished by simply replacing the coding sequences for extender module six with those for an extender module having a methylmalonyl specific AT and only a KR domain from a heterologous PKS gene, such as,

for example, the coding sequences for extender module two encoded by the *eryAI* gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that has a C-18 hydroxyl group. Such analogs are preferred neurotrophins, because they have little or no immunosuppressant activity. This recombinant sixth extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this sixth extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (having a C-18 hydroxyl group) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the DH and ER domains of module 6 have been deleted or otherwise rendered inactive and thus produces this novel polyketide.

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The seventh extender module of the FK-520 PKS includes a KS, an AT specific for 2-hydroxymalonyl CoA, a KR, a DH, an ER, and an ACP. The recombinant DNA compounds of the invention that encode the seventh extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 seventh extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the seventh extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encod the seventh extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion or all of the seventh extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the 2-hydroxymalonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or

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maionyl CoA specific AT: deleting the KR, the DH, and or the ER; and/or replacing the KR, DH, and/or ER. In addition, the KS and or ACP can be replaced with another KS and or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous seventh extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the seventh extender module of the FK-520 PKS.

In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the AT domain of the seventh extender module has been replaced with those encoding an AT domain for malonyl, methylmalonyl, or ethylmalonyl CoA from another PKS gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that lacks the C-15 methoxy group, having instead a hydrogen, methyl, or ethyl group at that position, respectively. Such analogs are preferred, because they are more slowly metabolized than FK-520. This recombinant seventh extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this seventh extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (C-15-desmethoxy) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the AT domain of module 7 has been replaced and thus produces this novel polyketide.

In another illustrative embodiment, the present invention provides a hybrid PKS in which the AT and KR domains of module 7 of the FK-520 PKS are replaced by a methylmalonyl specific AT domain and an inactive KR domain, such as, for example, the AT and KR domains of extender module 6 of the rapamycin PKS. The resulting hybrid PKS produces 15-desmethoxy-15-methyl-16-oxo-FK-520, a neurotrophin compound.

The eighth extender module of the FK-520 PKS includes a KS, an AT specific for 2-hydroxymalonyl CoA, a KR, and an ACP. The recombinant DNA compounds of the invention that encode the eighth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 eighth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the eighth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, pr.: ides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the eighth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

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In another embodiment, a portion of the eighth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the 2-hydroxymalonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or malonyl CoA specific AT; deleting or replacing the KR; and/or inserting a DH or a DH and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous eighth extender module coding sequence can be utilized in conjunction with a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the eighth extender module of the FK-520 PKS.

In an illustrative embodiment, the present invention provides a set of recombinant FK-520 PKS genes in which the coding sequences for the AT domain of the eighth extender module has been replaced with those encoding an AT domain for malonyl, methylmalonyl, or ethylmalonyl CoA from another PKS gene. The resulting PKS genes code for the expression of an FK-520 PKS that produces an FK-520 analog that lacks the C-13 methoxy group, having instead a hydrogen, methyl, or ethyl group at that position, respectively. Such

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analogs are preferred, because they are more slowly metabolized than FK-520. This recombinant eighth extender module coding sequence can be combined with other coding sequences to make additional compounds of the invention. In an illustrative embodiment, the present invention provides a recombinant FK-520 PKS that contains both this eighth extender module and the recombinant fourth extender module described above that comprises the coding sequence for the fourth extender module AT domain of the FK-506 PKS. The invention also provides recombinant host cells derived from FK-506 producing host cells that have been mutated to prevent production of FK-506 but that express this recombinant PKS and so synthesize the corresponding (C-13-desmethoxy) FK-506 derivative. In another embodiment, the present invention provides a recombinant FK-506 PKS in which the AT domain of module 8 has been replaced and thus produces this novel polyketide.

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The ninth extender module of the FK-520 PKS includes a KS, an AT specific for methylmalonyl CoA, a KR, a DH, an ER, and an ACP. The recombinant DNA compounds of the invention that encode the ninth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 ninth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the ninth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the ninth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion of the ninth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the methylmalonyl CoA specific AT with a malonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; deleting any one, two, or all three of the KR, DH, and ER: and/or replacing any one, two, or all three of the KR, DH, and ER with another KR, DH, and/or ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can

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originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous ninth extender module coding sequence can be utilized in conjunction with a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a module of a heterologous PKS can be replaced by one or more domains of the ninth extender module of the FK-520 PKS.

The tenth extender module of the FK-520 PKS includes a KS, an AT specific for malony! Come is an ACP. The recombinant DNA compounds of the invention that encode the tenth extender module of the FK-520 PKS and the corresponding polypeptides encoded thereby are useful for a variety of applications. In one embodiment, a DNA compound comprising a sequence that encodes the FK-520 tenth extender module is inserted into a DNA compound that comprises the coding sequence for a heterologous PKS. The resulting construct, in which the coding sequence for a module of the heterologous PKS is either replaced by that for the tenth extender module of the FK-520 PKS or the latter is merely added to coding sequences for the modules of the heterologous PKS, provides a novel PKS coding sequence. In another embodiment, a DNA compound comprising a sequence that encodes the tenth extender module of the FK-520 PKS is inserted into a DNA compound that comprises the coding sequence for the remainder of the FK-520 PKS or a recombinant FK-520 PKS that produces an FK-520 derivative.

In another embodiment, a portion or all of the tenth extender module coding sequence is utilized in conjunction with other PKS coding sequences to create a hybrid module. In this embodiment, the invention provides, for example, either replacing the malonyl CoA specific AT with a methylmalonyl CoA, ethylmalonyl CoA, or 2-hydroxymalonyl CoA specific AT; and/or inserting a KR, a KR and DH, or a KR, DH, and an ER. In addition, the KS and/or ACP can be replaced with another KS and/or ACP. In each of these replacements or insertions, the heterologous KS, AT, DH, KR, ER, or ACP coding sequence can originate from a coding sequence for another module of the FK-520 PKS, from a coding sequence for a PKS that produces a polyketide other than FK-520, or from chemical synthesis. The resulting heterologous tenth extender module coding sequence can be utilized in conjunction with a coding sequence for a PKS that synthesizes FK-520, an FK-520 derivative, or another polyketide. In similar fashion, the corresponding domains in a

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module of a heterologous PKS can be replaced by one or more domains of the tenth extender module of the FK-520 PKS.

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The FK-520 polyketide precursor produced by the action of the tenth extender module of the PKS is then attached to pipecolic acid and cyclized to form FK-520. The enzyme FkbP is the NRPS like enzyme that catalyzes these reactions. FkbP also includes a thioesterase activity that cleaves the nascent FK-520 polyketide from the NRPS. The present invention provides recombinant DNA compounds that encode the fkbP gene and so provides recombinant methods for expressing the fkbP gene product in recombinant host cells. The recombinant jkbP genes of the invention include those in which the coding sequence for the adenviation domain has been mutated or replaced with coding sequences from other NRPS like enzymes so that the resulting recombinant FkbP incorporates a moiety other than pipecolic acid. For the construction of host cells that do not naturally produce pipecolic acid, the present invention provides recombinant DNA compounds that express the enzymes that catalyze at least some of the biosynthesis of pipecolic acid (see Nielsen et al., 1991, Biochem. 30: 5789-96). The fkbL gene encodes a homolog of RapL, a lysine cyclodeaminase responsible in part for producing the pipecolate unit added to the end of the polyketide chain. The fkbB and fkbL recombinant genes of the invention can be used in heterologous hosts to produce compounds such as FK-520 or, in conjunction with other PKS or NRPS genes, to produce known or novel polyketides and non-ribosmal peptides.

The present invention also provides recombinant DNA compounds that encode the P450 oxidase and methyltransferase genes involved in the biosynthesis of FK-520. Figure 2 shows the various sites on the FK-520 polyketide core structure at which these enzymes act. By providing these genes in recombinant form, the present invention provides recombinant host cells that can produce FK-520. This is accomplished by introducing the recombinant PKS, P450 oxidase, and methyltransferase genes into a heterologous host cell. In a preferred embodiment, the heterologous host cell is *Strep.omyces coelicolor* CH999 or *Streptomyces lividans* K4-114, as described in U.S. Patent No. 5,830,750 and U.S. patent application Serial Nos. 08/828,898, filed 31 Mar. 1997, and 09/181,833, filed 28 Oct. 1998, each of which is incorporated herein by reference. In addition, by providing recombinant host cells that express only a subset of these genes, the present invention provides methods for making FK-520 precursor compounds not readily obtainable by other means.

In a related aspect, the present invention provides recombinant DNA compounds and vectors that are useful in generating, by homologous recombination, recombinant host

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cells that produce FK-520 precursor compounds. In this aspect of the invention, a native host cell that produces FK-520 is transformed with a vector (such as an SCP2* derived vector for *Streptomyces* host cells) that encodes one or more disrupted genes (i.e., a hydroxylase, a methyltransferase, or both) or merely flanking regions from those genes.

When the vector integrates by homologous recombination, the native, functional gene is deleted or replaced by the non-functional recombinant gene, and the resulting host cell thus produces an FK-520 precursor. Such host cells can also be complemented by introduction of a modified form of the deleted or mutated non-functional gene to produce a novel compound.

In one important embodiment, the present invention provides a hybrid PKS and the corresponding recombinant DNA compounds that encode those hybrid PKS enzymes. For purposes of the present invention a hybrid PKS is a recombinant PKS that comprises all or part of one or more modules and thioesterase cyclase domain of a first PKS and all or part of one or more modules, loading module, and thioesterase cyclase domain of a second PKS. In one preferred embodiment, the first PKS is all or part of the FK-520 PKS, and the second PKS is only a portion or all of a non-FK-520 PKS.

One example of the preferred embodiment is an FK-520 PKS in which the AT domain of module 8, which specifies a hydroxymalonyl CoA and from which the C-13 methoxy group of FK-520 is derived, is replaced by an AT domain that specifies a malonyl, methylmalonyl, or ethylmalonyl CoA. Examples of such replacement AT domains include the AT domains from modules 3, 12, and 13 of the rapaymycin PKS and from modules 1 and 2 of the erythromycin PKS. Such replacements, conducted at the level of the gene for the PKS, are illustrated in the examples below. Another illustrative example of such a hybrid PKS includes an FK-520 PKS in which the natural loading module has been replaced with a loading module of another PKS. Another example of such a hybrid PKS is an FK-520 PKS in which the AT domain of module three is replaced with an AT domain that binds methylmalonyl CoA.

In another preferred embodiment, the first PKS is most but not all of a non-FK-520 PKS, and the second PKS is only a portion or all of the FK-520 PKS. An illustrative example of such a hybrid PKS includes an erythromycin PKS in which an AT specific for methylmalonyl CoA is replaced with an AT from the FK-520 PKS specific for malonyl CoA.

Those of skill in the art will recognize that all or part of either the first or second PKS in a hybrid PKS of the invention need not be isolated from a naturally occurring source. For example, only a small portion of an AT domain determines its specificity. See U.S. provisional patent application Serial No. 60.091,526, incorporated herein by reference.

The state of the art in DNA synthesis allows the artisan to construct *de novo* DNA compounds of size sufficient to construct a useful portion of a PKS module or domain. For purposes of the present invention, such synthetic DNA compounds are deemed to be a portion of a PKS.

Thus, the hybrid modules of the invention are incorporated into a PKS to provide a hybrid PKS of the invention. A hybrid PKS of the invention can result not only:

- (i) from fusions of heterologous domain (where heterologous means the domains in that module are from at least two different naturally occurring modules) coding sequences to produce a hybrid module coding sequence contained in a PKS gene whose product is incorporated into a PKS.
- 15 but also:

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- (ii) from fusions of heterologous module (where heterologous module means two modules are adjacent to one another that are not adjacent to one another in naturally occurring PKS enzymes) coding sequences to produce a hybrid coding sequence contained in a PKS gene whose product is incorporated into a PKS,
- (iii) from expression of one or more FK-520 PKS genes with one or more non-FK-520 PKS genes, including both naturally occurring and recombinant non-FK-520 PKS genes, and
- (iv) from combinations of the foregoing.

 Various hybrid PKSs of the invention illustrating these various alternatives are described herein.

Examples of the production of a hybrid PKS by co-expression of PKS genes from the FK-520 PKS and another non-FK-520 PKS include hybrid PKS enzymes produced by coexpression of FK-520 and rapamycin PKS genes. Preferably, such hybrid PKS enzymes are produced in recombinant *Streptomyces* host cells that produce FK-520 or FK-506 but have been mutated to inactivate the gene whose function is to be replaced by the rapamycin PKS gene introduced to produce the hybrid PKS. Particular examples include (i) replacement of the *fkbC* gene with the *rapB* gene; and (ii) replacement of the *fkbA* gene with the *rapC* gene. The latter hybrid PKS produces 13,15-didesmethoxy-FK-520, if the host cell

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is an FK-520 producing host cell, and 13,15-didesmethoxy-FK-506, if the host cell is an FK-506 producing host cell. The compounds produced by these hybrid PKS enzymes are immunosuppressants and neurotrophins but can be readily modified to act only as neurotrophins, as described in Example 6, below.

Other illustrative hybrid PKS enzymes of the invention are prepared by replacing the fkbA gene of an FK-520 or FK-506 producing host cell with a hybrid fkbA gene in which: (a) the extender module 8 through 10, inclusive, coding sequences have been replaced by the coding sequences for extender modules 12 to 14, inclusive, of the rapamycin PKS; and (b) the module 8 coding sequences have been replaced by the module 8 coding sequence of the rifan Lin PKS. When expressed with the other, naturally occurring FK-520 or FK-506 PKS genes and the genes of the modification enzymes, the resulting hybrid PKS enzymes produce, respectively, (a) 13-desmethoxy-FK-520 or 13-desmethoxy-FK-506; and (b) 13desmethoxy-13-methyl-FK-520 or 13-desmethoxy-13-methyl-FK-506. In a preferred embodiment, these recombinant PKS genes of the invention are introduced into the producing host cell by a vector such as pHU204, which is a plamsid pRM5 derivative that has the well-characterized SCP2* replicon, the colE1 replicon, the tsr and bla resistance genes, and a cos site. This vector can be used to introduce the recombinant fkbA replacement gene in an FK-520 or FK-506 producing host cell (or a host cell derived therefrom in which the endogenous fkbA gene has either been rendered inactive by mutation, deletion or homologous recombination with the gene that replaces it) to produce the desired hybrid PKS.

In constructing hybrid PKSs of the invention, certain general methods may be helpful. For example, it is often beneficial to retain the framework of the module to be altered to make the hybrid PKS. Thus, if one desires to add DH and ER functionalities to a module, it is often preferred to replace the KR domain of the original module with a KR, DH, and ER domain-containing segment from another module, instead of merely inserting DH and ER domains. One can alter the stereochemical specificity of a module by replacement of the KS domain with a KS domain from a module that specifies a different stereochemistry. See Lau et al., 1999, "Dissecting the role of acyltransferase domains of modular polyketide synthases in the choice and stereochemical fate of extender units," *Biochemistry* 38(5):1643-1651, incorporated herein by reference. Stereochemistry can also be changed by changing the KR domain. Also, one can alter the specificity of an AT domain by changing only a small segment of the domain. See Lau et al., supra. One can

also take advantage of known linker regions in PKS proteins to link modules from two different PKSs to create a hybrid PKS. See Gokhale et al., 16 Apr. 1999, "Dissecting and Exploiting Intermodular Communication in Polyketide Synthases," *Science 284*: 482-485, incorporated herein by reference.

The following Table lists references describing illustrative PKS genes and corresponding enzymes that can be utilized in the construction of the recombinant PKSs and the corresponding DNA compounds that encode them of the invention. Also presented are various references describing tailoring enzymes and corresponding genes that can be employed in accordance with the methods of the sent invention.

10 Avermectin

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U.S. Pat. No. 5.252,474 to Merck.

MacNeil et al., 1993, Industrial Microorganisms: Basic and Applied Molecular Genetics, Baltz, Hegeman, & Skatrud, eds. (ASM), pp. 245-256, A Comparison of the Genes Encoding the Polyketide Synthases for Avermectin, Erythromycin, and Nemadectin.

MacNeil et al., 1992, Gene 115: 119-125, Complex Organization of the Streptomyces avermittilis genes encoding the avermectin polyketide synthase.

Ikeda *et al.*. Aug. 1999, Organization of the biosynthetic gene cluster for the polyketide anthelmintic macrolide avermectin in *Streptomyces avermitilis*, *Proc. Natl. Acad. Sci. USA* 96: 9509-9514.

20 Candicidin (FR008)

Hu et al., 1994, Mol. Microbiol. 14: 163-172.

Epothilone

U.S. Pat. App. Serial No. 60/130,560, filed 22 April 1999.

Erythromycin

25 PCT Pub. No. 93/13663 to Abbott.

US Pat. No. 5.824,513 to Abbott.

Donadio et al., 1991, Science 252:675-9.

Cortes et al., 8 Nov. 1990, Nature 348:176-8, An unusually large multifunctional polypeptide in the erythromycin producing polyketide synthase of Saccharopolyspora erythraea.

Glycosylation Enzymes

PCT Pat. App. Pub. No. 97/23630 to Abbott.

FK-506

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Motamedi *et al.*, 1998. The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK-506, *Eur. J. biochem.* 256; 528-534.

Motamedi *et al.*, 1997, Structurai organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK-506, *Eur. J. Biochem.* 244: 74-80.

Methyltransferase

US 5,264,355, issued 23 Nov. 1993, Methylating enzyme from *Streptomyces* MA6858, 31-O-desmethyl-FK-506 methyltransferase.

Motamedi *et al.*, 1996. Characterization of methyltransferase and hydroxylase genes involved in the biosynthesis of the immunosuppressants FK-506 and FK-520, *J. Bacteriol.* 178: 5243-5248.

Streptomyces hygroscopicus

U.S. patent application Serial No. 09/154,083, filed 16 Sep. 1998.

Lovastatin

U.S. Pat. No. 5,744,350 to Merck.

Narbomycin

U.S. patent application Serial No. 60/107.093, filed 5 Nov. 1998, and Serial No. 60/120,254, filed 16 Feb. 1999.

Nemadectin

20 MacNeil et al., 1993, supra.

Niddamycin

Kakavas *et al.*, 1997. Identification and characterization of the niddamycin polyketide synthase genes from *Streptomyces caelestis*, *J. Bacteriol.* 179: 7515-7522.

Oleandomycin

Swan *et al.*, 1994, Characterisation of a *Streptomyces antibioticus* gene encoding a type I polyketide synthase which has an unusual coding sequence, *Mol. Gen. Genet.* 242: 358-362.

U.S. patent application Serial No. 60/120,254, filed 16 Feb. 1999.

Olano et al., 1998. Analysis of a *Streptomyces antibioticus* chromosomal region involved in oleandomycin biosynthesis, which encodes two glycosyltransferases responsible for glycosylation of the macrolactone ring, *Mol. Gen. Genet.* 259(3): 299-308.

Picromycin

PCT patent application US99/15047, filed 2 Jul. 1999.

Xue *et al.*, 1998, Hydroxylation of macrolactones YC-17 and narbomycin is mediated by the *pik*C-encoded cytochrome P450 in *Streptomyces venezuelae*, *Chemistry & Biology* 5(11): 061-667.

Xue et al., Oct. 1998, A gene cluster for macrolide antibiotic biosynthesis in

Streptomyces venezuelae: Architecture of metabolic diversity, Proc. Natl. Acad. Sci. USA

95: 12111-12116.

Platenolide

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EP Pat. App. Pub. No. 791,656 to Lilly.

Papamycin

Schwecke *et al.*, Aug. 1995, The biosynthetic gene cluster for the polyketide rapamycin, *Proc. Natl. Acad. Sci. USA 92:*7839-7843.

Aparicio et al., 1996, Organization of the biosynthetic gene cluster for rapamycin in Streptomyces hygroscopicus: analysis of the enzymatic domains in the modular polyketide synthase, Gene 169: 9-16.

15 Rifamycin

August *et al.*, 13 Feb. 1998, Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S669, *Chemistry & Biology*, 5(2): 69-79.

Sorangium PKS

U.S. patent application Serial No. 09/144,085, filed 31 Aug. 1998.

Soraphen

U.S. Pat. No. 5,716,849 to Novartis.

Schupp et al., 1995, J. Bacteriology 177: 3673-3679. A Sorangium cellulosum (Myxobacterium) Gene Cluster for the Biosynthesis of the Macrolide Antibiotic Soraphen

A: Cloning, Characterization, and Homology to Polyketide Synthase Genes from Actinomycetes.

Spiramycin

U.S. Pat. No. 5,098,837 to Lilly.

Activator Gene

30 U.S. Pat. No. 5,514,544 to Lilly.

Tylosin

EP Pub. No. 791,655 to Lilly.

U.S. Pat. No. 5,876,991 to Lilly.

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Kuhstoss et al., 1996, Gene 183:231-6.. Production of a novel polyketide through the construction of a hybrid polyketide synthase.

Tailoring enzymes

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Merson-Davies and Cundliffe, 1994, Mol. Microbiol. 13: 349-355. Analysis of five tylosin biosynthetic genes from the tylBA region of the Streptomyces fradiae genome.

As the above Table illustrates, there are a wide variety of polyketide synthase genes that serve as readily available sources of DNA and sequence information for use in constructing the hybrid PKS-encoding DNA compounds of the invention. Methods for constructing hybrid PKS-encoding DNA compounds are described without reference to the FK-520 PKS in PCT patent publication No. 98-51695; U.S. Patent Nos. 5,672,491 and 5,712,146 and U.S. patent application Serial Nos. 09/073,538, filed 6 May 1998, and 09/141,908, filed 28 Aug 1998, each of which is incorporated herein by reference.

The hybrid PKS-encoding DNA compounds of the invention can be and often are hybrids of more than two PKS genes. Moreover, there are often two or more modules in the hybrid PKS in which all or part of the module is derived from a second (or third) PKS. Thus, as one illustrative example, the present invention provides a hybrid FK-520 PKS that contains the naturally occurring loading module and FkbP as well as modules one, two, four, six, seven, and eight, nine, and ten of the FK-520 PKS and further contains hybrid or heterologous modules three and five. Hybrid or heterologous module three contains an AT domain that is specific of methylmalonyl CoA and can be derived for example, from the erythromycin or rapamycin PKS genes. Hybrid or heterologous module five contains an AT domain that is specific for malonyl CoA and can be derived for example, from the picromycin or rapamycin PKS genes.

While an important embodiment of the present invention relates to hybrid PKS enzymes and corresponding genes, the present invention also provides recombinant FK-520 PKS genes in which there is no second PKS gene sequence present but which differ from the FK-520 PKS gene by one or more deletions. The deletions can encompass one or more modules and/or can be limited to a partial deletion within one or more modules. When a deletion encompasses an entire module, the resulting FK-520 derivative is at least two carbons shorter than the gene from which it was derived. When a deletion is within a module, the deletion typically encompasses a KR. DH, or ER domain, or both DH and ER domains, or both KR and DH domains, or all three KR, DH, and ER domains.

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To construct a hybrid PKS or FK-520 derivative PKS gene of the invention, one can employ a technique, described in PCT Pub. No. 98/27203 and U.S. patent application Serial No. 08 989,332. Mied 11 Dec. 1997, each of which is incorporated herein by reference, in which the large PKS gene is divided into two or more, typically three, segments, and each segment is placed on a separate expression vector. In this manner, each of the segments of the gene can be altered, and various altered segments can be combined in a single host cell to provide a recombinant PKS gene of the invention. This technique makes more efficient the construction of large libraries of recombinant PKS genes, vectors for expressing those genes, and host cells comprising those vectors.

Thus, in one important embodiment, the recombinant DNA compounds of the invention are expression vectors. As used herein, the term expression vector refers to any nucleic acid that can be introduced into a host cell or cell-free transcription and translation medium. An expression vector can be maintained stably or transiently in a cell, whether as part of the chromosomal or other DNA in the cell or in any cellular compartment, such as a replicating vector in the cytoplasm. An expression vector also comprises a gene that serves to produce RNA that is translated into a polypeptide in the cell or cell extract. Furthermore, expression vectors typically contain additional functional elements, such as resistance-conferring genes to act as selectable markers.

The various components of an expression vector can vary widely, depending on the intended use of the vector. In particular, the components depend on the host cell(s) in which the vector will be used or is intended to function. Vector components for expression and maintenance of vectors in *E. coli* are widely known and commercially available, as are vector components for other commonly used organisms, such as yeast cells and *Streptomyces* cells.

In a preferred embodiment, the expression vectors of the invention are used to construct recombinant *Streptomyces* host cells that express a recombinant PKS of the invention. Preferred *Streptomyces* host cell/vector combinations of the invention include *S. coelicolor* CH999 and *S. lividans* K4-114 host cells, which do not produce actinorhodin, and expression vectors derived from the pRM1 and pRM5 vectors, as described in U.S. Patent No. 5,830,750 and U.S. patent application Serial Nos. 08/828,898, filed 31 Mar. 1997, and 09/181,833, filed 28 Oct. 1998, each of which is incorporated herein by reference.

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The present invention provides a wide variety of expression vectors for use in Streptomyces. For replicating vectors, the origin of replication can be, for example and without limitation, a low copy number vector, such as SCP2* (see Hopwood et al., Genetic Manipulation of Streptomyces: A Laboratory manual (The John Innes Foundation, 5 Norwich, U.K., 1985); Lydiate et al., 1985, Gene 35: 223-235; and Kieser and Melton, 1988, Gene 65: 83-91, each of which is incorporated herein by reference), SLP1.2 (Thompson et al., 1982, Gene 20: 51-62, incorporated herein by reference), and SG5(ts) (Muth et al., 1989, Mol. Gen. Genet. 219: 341-348, and Bierman et al., 1992, Gene 116: 43-49, each of which is incorporated herein by reference), or a high copy number vector, such 10 as pIJ101 and pJV1 (see Katz et al., 1983, J. Gen. Microbio). 29: 2703-2714; Var., et al., 1989, J. Bacteriol. 171: 5782-5781; and Servin-Gonzalez. 1993, Plasmid 30: 131-140, each of which is incorporated herein by reference). Generally, however, high copy number vectors are not preferred for expression of genes contained on large segments of DNA. For non-replicating and integrating vectors, it is useful to include at least an E. coli origin of 15 replication, such as from pUC, p1P, p1I, and pBR. For phage based vectors, the phages phiC31 and KC515 can be employed (see Hopwood et al., supra).

Typically, the expression vector will comprise one or more marker genes by which host cells containing the vector can be identified and/or selected. Useful antibiotic resistance conferring genes for use in *Streptomyces* host cells include the *ermE* (confers resistance to erythromycin and other macrolides and lincomycin). *tsr* (confers resistance to thiostrepton), *aadA* (confers resistance to spectinomycin and streptomycin), *aacC4* (confers resistance to apramycin, kanamycin, gentamicin, geneticin (G418), and neomycin), *hyg* (confers resistance to hygromycin), and *vph* (confers resistance to viomycin) resistance conferring genes.

The recombinant PKS gene on the vector will be under the control of a promoter, typically with an attendant ribosome binding site sequence. The present invention provides the endogenous promoters of the FK-520 PKS and related biosynthetic genes in recombinant form, and these promoters are preferred for use in the native hosts and in heterologous hosts in which the promoters function. A preferred promoter of the invention is the *fkbO* gene promoter, comprised in a sequence of about 270 bp between the start of the open reading frames of the *fkbO* and *fkbB* genes. The *fkbO* promoter is believed to be bidirectional in that it promotes transcription of the genes *fkbO*, *fkbP*, and *fkbA* in one direction and *fkbB*, *fkbC*, and *fkbL* in the other. Thus, in one aspect, the present invention

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provides a recombinant expression vector comprising the promoter of the *fkbO* gene of an FK-520 producing organism positioned to transcribe a gene other than *fkbO*. In a preferred embodiment the transcribed gene is an FK-520 PKS gene. In another preferred embodiment, the transcribed gene is a gene that encodes a protein comprised in a hybrid PKS.

Heterologous promoters can also be employed and are preferred for use in host cells in which the endogenous FK-520 PKS gene promoters do not function or function poorly. A preferred heterologous promoter is the actI promoter and its attendant activator gene actII-ORF4, which is provided in the pRM1 and pRM5 expression vectors, supra. This promoter is activated but stationary phase of growth when secondary metabolites are normalisynthesized. Other useful Streptomyces promoters include without limitation those from the ermE gene and the melC1 gene, which act constitutively, and the tipA gene and the merA gene, which can be induced at any growth stage. In addition, the T7 RNA polymerase system has been transferred to Streptomyces and can be employed in the vectors and host cells of the invention. In this system, the coding sequence for the T7 RNA polymerase is inserted into a neutral site of the chromosome or in a vector under the control of the inducible merA promoter, and the gene of interest is placed under the control of the T7 promoter. As noted above, one or more activator genes can also be employed to enhance the activity of a promoter. Activator genes in addition to the actII-ORF4 gene discussed above include dnrl, redD, and ptpA genes (see U.S. patent application Serial No. 09/181,833, supra) to activate promoters under their control.

In addition to providing recombinant DNA compounds that encode the FK-520 PKS, the present invention also provides DNA compounds that encode the ethylmalonyl CoA and 2-hydroxymalonyl CoA utilized in the synthesis of FK-520. Thus, the present invention also provides recombinant host cells that express the genes required for the biosynthesis of ethylmalonyl CoA and 2-hydroxymalonyl CoA. Figures 3 and 4 show the location of these genes on the cosmids of the invention and the biosynthetic pathway that produces ethylmalonyl CoA.

For 2-hydroxymalonyl CoA biosynthesis, the fkbH, fkbI, fkbJ, and fkbK genes are sufficient to confer this ability on Streptomcyces host cells. For conversion of 2-hydroxymalonyl to 2-methoxymalonyl, the fkbG gene is also employed. While the complete coding sequence for fkbH is provided on the cosmids of the invention, the sequence for this gene provided herein may be missing a T residue, based on a comparison made with a similar gene cloned from the ansamitocin gene cluster by Dr. H. Floss. Where the sequence

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herein shows one T, there may be two, resulting in an extension of the *fkbH* reading frame to encode the amino acid sequence:

MTIVKCI.VWDLDNTLWRGTVLEDDEVVLTDEIREVITTLDDRGILQAVASKNDHD LAWERLERLGVAEYFVLARIGWGPKSQSVREIATELNFAPTTIAFIDDQPAERAEVA FHLPEVRCYPAEQAATLLSLPEFSPPVSTVDSRRRRLMYQAGFARDQAREAYSGPD EDFLRSLDLSMTIAPAGEEELSRVEELTLRTSQMNATGVHYSDADLRALLTDPAHE VLVVTMGDRFGPHGAVGIILLEKKPSTWHLKLLATSCRVVSFGAGATILNWLTDQG ARAGAHLVADFRRTDRNRMMEIAYRFAGFADSDCPCVSEVAGASAAGVERLHLEP SARPAPTTLTLTAADIAPVTVSAAG.

For ethylmalonyl CoA biosynthesis, one requires only a crotonyl CoA reductase, which can be supplied by the host cell but can also be supplied by recombinant expression of the *fkbS* gene of the present invention. To increase yield of ethylmalonyl CoA, one can also express the *fkbE* and *fkbU* genes as well. While such production can be achieved using only the recombinant genes above, one can also achieve such production by placing into the recombinant host cell a large segment of the DNA provided by the cosmids of the invention. Thus, for 2-hydroxymalonyl and 2-methoxymalonyl CoA biosynthesis, one can simply provide the cells with the segment of DNA located on the left side of the FK-520 PKS genes shown in Figure 1. For ethylmalonyl CoA biosynthesis, one can simply provide the cells with the segment of DNA located on the right side of the FK-520 PKS genes shown in Figure 1 or, alternatively, both the right and left segments of DNA.

The recombinant DNA expression vectors that encode these genes can be used to construct recombinant host cells that can make these important polyketide building blocks from cells that otherwise are unable to produce them. For example, *Streptomyces coelicolor* and *Streptomyces lividans* do not synthesisze ethylmalonyl CoA or 2-hydroxymalonyl CoA. The invention provides methods and vectors for constructing recombinant *Streptomyces coelicolor* and *Streptomyces lividans* that are able to synthesize either or both ethylmalonyl CoA and 2-hydroxymalonyl CoA. These host cells are thus able to make polyketides, those

In a preferred embodiment, the present invention provides recombinant Streptomyces host cells, such as S. coelicolor and S. lividans, that have been transformed with a recombinant vector of the invention that codes for the expression of the ethylmalonyl CoA biosynthetic genes. The resulting host cells produce ethylmalonyl CoA and so are preferred host cells for the production of polyketides produced by PKS enzymes that

requiring these substrates, that cannot otherwise be made in such cells.

comprise one or more AT domains specific for ethylmalonyl CoA. Illustrative PKS enzymes of this type include the FK-520 PKS and a recombinant PKS in which one or more AT domains is specific for ethylmalonyl CoA.

In a related embodiment, the present invention provides *Streptomyces* host cells in which one or more of the ethylmalonyl or 2-hydroxymalonyl biosynthetic genes have been deleted by homologous recombination or rendered inactive by mutation. For example, deletion or inactivation of the *fkbG* gene can prevent formation of the methoxyl groups at C-13 and C-15 of FK-520 (or, in the corresponding FK-506 producing cell, FK-506), leading to the production of 13,15-didesmethoxy-13,15-dihydroxy-FK-520 (or, in the corresponding FK-506 producing cell, 13,15-didesmethoxy-13,15-dihydroxy-FK-506). If the *fkbG* gene product acts on 2-hydroxymalonyl and the resulting 2-methoxymalonyl substrate is required for incorporation by the PKS, the AT domains of modules 7 and 8 may bind malonyl CoA and methylmalonyl CoA. Such incorporation results in the production of a mixture of polyketides in which the methoxy groups at C-13 and C-15 of FK-520 (or FK-506) are replaced by either hydrogen or methyl.

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This possibility of non-specific binding results from the construction of a hybrid PKS of the invention in which the AT domain of module 8 of the FK-520 PKS replaced the AT domain of module 6 of DEBS. The resulting PKS produced, in *Streptomyces lividans*, 6-dEB and 2-desmethyl-6-dEB, indicating that the AT domain of module 8 of the FK-520 PKS could bind malonyl CoA and methylmalonyl CoA substrates. Thus, one could possibly also prepare the 13.15-didesmethoxy-FK-520 and corresponding FK-506 compounds of the invention by deleting or otherwise inactivating one or more or all of the genes required for 2-hydroxymalonyl CoA biosynthesis, i.e., the *fkbH*, *fkbI*, *fkbJ*, and *fkbK* genes. In any event, the deletion or inactivation of one or more biosynthetic genes required for ethylmalonyl and/or 2-hydroxymalonyl production prevents the formation of polyketides requiring ethylmalonyl and/or 2-hydroxymalonyl for biosynthesis, and the resulting host cells are thus preferred for production of polyketides that do not require the same.

The host cells of the invention can be grown and fermented under conditions known in the art for other purposes to produce the compounds of the invention. See, e.g., U.S. Patent Nos. 5,194,378; 5,116,756; and 5,494,820, incorporated herein by reference, for suitable fermentation processes. The compounds of the invention can be isolated from the fermentation broths of these cultured cells and purified by standard procedures. Preferred compounds of the invention include the following compounds: 13-desmethoxy-FK-506; 13-

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desmethoxy-FK-520; 13,15-didesmethoxy-FK-506; 13.15-didesmethoxy-FK-520; 13-desmethoxy-18-hydroxy-FK-506; 13-desmethoxy-18-hydroxy-FK-520; 13,15-didesmethoxy-18-hydroxy-FK-500; and 13,15-didesmethoxy-18-hydroxy-FK-520. These compounds can be further modified as described for tacrolimus and FK-520 in U.S. Patent Nos. 5,225,403; 5,189,042; 5,164,495; 5,068,323; 4,980,400; and 4,920,218, incorporated herein by reference.

Other compounds of the invention are shown in Figure 8, Parts A and B. In Figure 8, Part A, illustrative C-32-substituted compounds of the invention are shown in two columns under the heading R. The substituted compounds are preferred for topical administration and are applied to the dermis for treatment—conditions such as psoriasis. In Figure 8, Part B, illustrative reaction schemes for making the compounds shown in Figure 8, Part A, are provided. In the upper scheme in Figure 8, Part B, the C-32 substitution is a tetrazole moiety, illustrative of the groups shown in the left column under R in Figure 8, Part A. In the lower scheme in Figure 8, Part B, the C-32 substitution is a disubstituted amino group, where R₃ and R₄ can be any group similar to the illustrative groups shown attached to the amine in the right column under R in Figure 8. Part A. While Figure 8 shows the C-32-substituted compounds in which the C-15-methoxy is present, the invention includes these C-32-substituted compounds in which C-15 is ethyl, methyl, or hydrogen. Also, while C-21 is shown as substituted with ethyl or allyl, the compounds of the invention includes the C-32-substituted compounds in which C-21 is substituted with hydrogen or methyl.

To make these C-32-substituted compounds, Figure 8, Part B, provides illustrative reaction schemes. Thus, a selective reaction of the starting compound (see Figure 8, Part B, for an illustrative starting compound) with trifluoromethanesulfonic anhydride in the presence of a base yields the C-32 O-triflate derivative, as shown in the upper scheme of Figure 8, Part B. Displacement of the triflate with 1H-tetrazole or triazole derivatives provides the C-32 tetrazole or teiazole derivative. As shown in the lower scheme of Figure 8, Part B, reacting the starting compound with p-nitrophenylchloroformate yields the corresponding carbonate, which, upon displacement with an amino compound, provides the corresponding carbamate derivative.

The compounds can be readily formulated to provide the pharmaceutical compositions of the invention. The pharmaceutical compositions of the invention can be used in the form of a pharmaceutical preparation, for example, in solid, semisolid, or liquid form. This preparation contains one or more of the compounds of the invention as an active

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ingredient in admixture with an organic or inorganic carrier or excipient suitable for external, enteral, or parenteral application. The active ingredient may be compounded, for example, with the usual non-toxic, pharmaceutically acceptable carriers for tablets, pellets, capsules, suppositories, solutions, emulsions, suspensions, and any other form suitable for use. Suitable formulation processes and compositions for the compounds of the present invention are described with respect to tacrolimus in U.S. Patent Nos. 5,939,427; 5,922,729; 5,385,907; 5,338,684; and 5,260,301, incorporated herein by reference. Many of the compounds of the invention contain one or more chiral centers, and all of the stereoisomers are included within the scope of the invention, as pure compounds as well as mixtures of stereoisomers. Thus the compounds of the invention may be supplied as a mixture of stereoisomers in any proportion.

The carriers which can be used include water, glucose, lactose, gum acacia, gelatin, mannitol, starch paste, magnesium trisilicate, tale, corn starch, keratin, colloidal silica, potato starch, urea, and other carriers suitable for use in manufacturing preparations, in solid, semi-solid, or liquified form. In addition, auxiliary stabilizing, thickening, and coloring agents and perfumes may be used. For example, the compounds of the invention may be utilized with hydroxypropyl methylcellulose essentially as described in U.S. Patent No. 4,916,138, incorporated herein by reference, or with a surfactant essentially as described in EPO patent publication No. 428,169, incorporated herein by reference.

Oral dosage forms may be prepared essentially as described by Hondo *et al.*, 1987, *Transplantation Proceedings XIX*, Supp. 6: 17-22, incorporated herein by reference. Dosage forms for external application may be prepared essentially as described in EPO patent publication No. 423,714, incorporated herein by reference. The active compound is included in the pharmaceutical composition in an amount sufficient to produce the desired effect upon the disease process or condition.

For the treatment of conditions and diseases relating to immunosuppression or neuronal damage, a compound of the invention may be administered orally, topically, parenterally, by inhalation spray, or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvant, and vehicles. The term parenteral, as used herein, includes subcutaneous injections, and intravenous, intramuscular, and intrasternal injection or infusion techniques.

Dosage levels of the compounds of the present invention are of the order from about 0.01 mg to about 50 mg per kilogram of body weight per day, preferably from about 0.1 mg

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to about 10 mg per kilogram of body weight per day. The dosage levels are useful in the treatment of the above-indicated conditions (from about 0.7 mg to about 3.5 mg per patient per day, assuming a 70 kg patient). In addition, the compounds of the present invention may be administered on an intermittent basis, i.e., at semi-weekly, weekly, semi-monthly, or monthly intervals.

The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a formulation intended for oral administration to humans may contain from 0.5 mg to 5 g of active agent compounded with an appropriate and convenient amount of carrier material, which may vary from about 5 percent to about 95 percent of the total composition. Dosage unit forms will generally contain from about 0.5 mg to about 500 mg of active ingredient. For external administration, the compounds of the invention can be formulated within the range of, for example, 0.00001% to 60% by weight, preferably from 0.001% to 10% by weight, and most preferably from about 0.005% to 0.8% by weight. The compounds and compositions of the invention are useful in treating disease conditions using doses and administration schedules as described for tacrolimus in U.S. Patent Nos. 5,542,436; 5,365,948; 5,348,966; and 5,196,437, incorporated herein by reference. The compounds of the invention can be used as single therapeutic agents or in combination with other therapeutic agents. Drugs that can be usefully combined with compounds of the invention include one or more immunosuppressant agents such as rapamycin, cyclosporin A, FK-506, or one or more neurotrophic agents.

It will be understood, however, that the specific dosage level for any particular patient will depend on a variety of factors. These factors include the activity of the specific compound employed; the age, body weight, general health, sex. and diet of the subject; the time and route of administration and the rate of excretion of the drug; whether a drug combination is employed in the treatment; and the severity of the particular disease or condition for which therapy is sought.

A detailed description of the invention having been provided above, the following examples are given for the purpose of illustrating the present invention and shall not be construed as being a limitation on the scope of the invention or claims.

Example 1

Replacement of Methoxvl with Hvdrogen or Methyl at C-13 of FK-520

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The C-13 methoxyl group is introduced into FK-520 via an AT domain in extender module 8 of the PKS that is specific for hydroxymalonyl and by methylation of the hydroxyl group by an S-adenosyl methionine (SAM) dependent methyltransferase. Metabolism of FK-506 and FK-520 primarily involves oxidation at the C-13 position into an inactive derivative that is further degraded by host P450 and other enzymes. The present invention provides compounds related in structure to FK-506 and FK-520 that do not contain the C-13 methoxy group and exhibit greater stability and a longer half-life *in vivo*. These compounds are useful medicaments due to their immunosuppressive and neurotrophic activities, and the invention provides the compounds in purified form and as pharmaceutical compositions.

The present invention also provides the novel PKS enzymes that produce these novel compounds as well as the expression vectors and host cells that produce the novel PKS enzymes. The novel PKS enzymes include, among others, those that contain an AT domain specific for either malonyl CoA or methylmalonyl CoA in module 8 of the FK-506 and FK-520 PKS. This example describes the construction of recombinant DNA compounds that encode the novel FK-520 PKS enzymes and the transformation of host cells with those recombinant DNA compounds to produce the novel PKS enzymes and the polyketides produced thereby.

To construct an expression cassette for performing module 8 AT domain replacements in the FK-520 PKS, a 4.6 kb *Sph*I fragment from the FK-520 gene cluster was cloned into plasmid pLitmus 38 (a cloning vector available from New England Biolabs). The 4.6 kb *Sph*I fragment, which encodes the ACP domain of module 7 followed by module 8 through the KR domain, was isolated from an agarose gel after digesting the cosmid pKOS65-C31 with *Sph* I. The clone having the insert oriented so the single *Sac*I site was nearest to the *Spe*I end of the polylinker was identified and designated as plasmid pKOS60-21-67. To generate appropriate cloning sites, two linkers were ligated sequentially as follows. First, a linker was ligated between the *Spe*I and *Sac*I sites to introduce a *Bgl*II site at the 5' end of the cassette, to eliminate interfering polylinker sites, and to reduce the total insert size to 4.5 kb (the limit of the phage KC515). The ligation reactions contained 5 picomolar unphosphorylated linker DNA and 0.1 picomolar vector DNA, i.e., a 50-fold molar excess of linker to vector. The linker had the following sequence:

5'-CTAGTGGGCAGATCTGGCAGCT-3' 3'-ACCCGTCTAGACCG-5'

The resulting plasmid was designated pKOS60-27-1.

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Next, a linker of the following sequence was ligated between the unique *Sph*I and *Afl*II sites of plasmid pKOS60-27-1 to introduce an *Nsi*I site at the 3' end of the module 8 cassette. The linker employed was:

5'-GGGATGCATGGC-3'
3'-GTACCCCTACGTACCGAATT-5'

The resulting plasmid was designated pKOS60-29-55.

To allow in-frame insertions of alternative AT domains, sites were engineered at the 5' end (Avr II or Nhe I) and 3' end (Aho I) of the AT domain using the polymerase chain reaction (PCR) as follows. Plasmid pKOS60-29-55 was used as a template for the PCR and sequence 5' to the AT domain was amplified with the primers SpeBgl-fwd and either Avr-rev or Nhe-rev:

SpeBgl-fwd 5'-CGACTCACTAGTGGGCAGATCTGG-3'

Avr-rev 5'-CACGCCTAGGCCGGTCGGTCTCGGGCCAC-3'

Nhe-rev 5'-GCGGCTAGCTGCTCGCCCATCGCGGGATGC-3'

The PCR included, in a 50 μl reaction, 5 μl of 10x Pfu polymerase buffer (Stratagene), 5 μl 10x z-dNTP mixture (2 mM dATP, 2 mM dCTP, 2 mM dTTP, 1 mM dGTP, 1 mM 7-deaza-GTP), 5 μl DMSO, 2 μl of each primer (10 μM), 1 μl of template DNA (0.1 μg/μl), and 1 μl of cloned Pfu polymerase (Stratagene). The PCR conditions were 95°C for 2 min., 25 cycles at 95°C for 30 sec., 60°C for 30 sec., and 72°C for 4 min., followed by 4 min. at 72°C and a hold at 0°C. The amplified DNA products and the Litmus vectors were cut with the appropriate restriction enzymes (Bg/II and AvrII or SpeI and NheI), and cloned into either pLitmus 28 or pLitmus38 (New England Biolabs), respectively, to generate the constructs designated pKOS60-37-4 and pKOS60-37-2, respectively.

Plasmid pKOS60-29-55 was again used as a template for PCR to amplify sequence 3' to the AT domain using the primers BsrXho-fwd and NsiAfl-rev:

BsrXho-fiwd 5'-GATGTACAGCTCGAGTCGGCACGCCCGGCCGCATC-3'
NsiAfl-rev 5'-CGACTCACTTAAGCCATGCATCC-3'

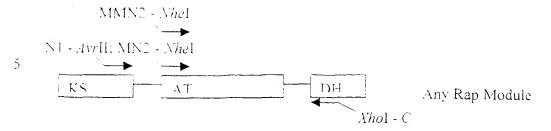
PCR conditions were as described above. The PCR fragment was cut with *BsrGI* and *AfIII*, gel isolated, and ligated into pKOS60-37-4 cut with *Asp*718 and *AfIII* and inserted into pKOS60-37-2 cut with *BsrGI* and *AfIII*, to give the plasmids pKOS60-39-1 and pKOS60-39-13, respectively. These two plasmids can be digested with *AvrII* and *XhoI* or *NheI* and *XhoI*, respectively, to insert heterologous AT domains specific for malonyl, methylmalonyl, ethylmalonyl, or other extender units.

Malonyl and methylmalonyl-specific AT domains were cloned from the rapamycin cluster using PCR amplification with a pair of primers that introduce an *AvrII* or *NheI* site at the 5' end and an *XhoI* site at the 3' end. The PCR conditions were as given above and the primer sequences were as follows:

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RATN1 5'-ATCCTAGGCGGGCRGGYGTGTCGTCGTCGG-3'
(3' end of Rap KS sequence and universal for malonyl and methylmalonyl CoA),
RATMN2 5'-ATGCTAGCCGCGCGTTCCCCGTCTTCGCGCG-3'
(Rap AT shorter version 5'- sequence and specific for malonyl CoA),
RATMMN2 5'-ATGCTAGCGATTCGTCGGTGGTGTTCGCCGA 3'

10 RATMMN2 5'-ATGCTAGCGGATTCGTCGGTGGTGTTCGCCGA-3' (Rap AT shorter version 5'- sequence and specific for methylmalonyl CoA), and RATC 5'-ATCTCGAGCCAGTASCGCTGGTGYTGGAAGG-3' (Rap DH 5'- sequence and universal for malonyl and methylmalonyl CoA).



Because of the high sequence similarity in each module of the rapamycin cluster, each primer was expected to prime any of the AT domains. PCR products representing ATs specific for malonyl or methylmalonyl extenders were identified by sequencing individual cloned ^r ^rR products. Sequencing also confirmed that the chosen clones contained no cloning artifacts. Examples of hybrid modules with the rapamycin AT12 and AT13 domains are shown in a separate figure.

The AvrII-AhoI restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 12 of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below. The AT of rap module 12 is specific for incorporation of malonyl units.

20 AGATOTGGCAGOTCGCCGAAGOGCTGCTGACGCTCGTCCGGGAGAGCACC 50 I W O L A E A L L T L V R E S T GCCGCCGTGCTCGGCCACGTGGGTGGCGAGGACATCCCCGCGACGCCGC 100 A A V L G H V G G E D I P A T A A GTTCAAGGACCTCGGCATCGACTCGCTCACCGCGGTCCAGCTGCGCAACG 150 25 F K D L G I D S L T A V Q L R N COCTCACOGAGGOGACOGGTGTGCGGCTGAACGCCACGGCGGTCTTCGAC 200 A L T E A T G V R L N A T A V F D TTOCOGRECOCGCACGTGCTCGCCGGGARGCTCGGCGACGAACTGACCGG 250 FPTPHVLAGELGPELTG 30 CACCCGCGCGCCCTCGTGCCCCGGACCGCGCCACGGCCGGTGCGCACG 300 T R A P V V P R T A A T A G A H ASGASCOGCTGGCGATCGTGGGGAATGGCCTGCCGGCTGCCCGGCGGGGGGTC 350 D E P L A I V G M A C E L P G G V GCGTCACCCGAGGAGCTSTGGCACCTCGTGGCATCCGGCACCGACGCCAT 400 35 A S P E E L W H L V A S G T D A CACGGAGTTCCCGACGGACGCGCTGGGACGTCGACGCGATCTACGACC 450 TEFFTDRGWDVDAIYD CGGACCCCGACGCGATCGGCAAGACCTTCGTCCGGCACGGTGGCTTCCTC 500 PDPDAIGKTFVRHGGFL 40 ACCGGCGCGACAGGCTTCGACGCGGCGTTCTTCGGCATCAGCCCGCGCGA 550 TGATGFDAAFFGISPRE GGCCCTCGCGATGGACCCGCAGCAGCGGGTGCTCCTGGAGACGTCGTGGG 600 A L A M D P Q Q R V L L E T S W AGGCGTTCGAAAGCGCCGGCATCACCCCGGACTCGACCCGCGGCAGCGAC 650 45 E A F E S A G I T P D S T R G S D ACCGCCGTGTTCGTCGGCCCTTCTCCTACGGTTACGGCACCGGTGCGGA 700 T G V F T G A F S Y G Y G T G A D OACCGACGGCTTCGGCGCGACCGGCTCGCAGACCAGTGTGCTCTCCGGCC 750 T D G F G A T G S Q T S V L S G 50 GGCTGTCGTACTTCTACGGTCTGGAGGGTCCGGCGGTCACGGTCGACACG 800

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TT not pato set astroact as reacat as Asaca egot bissak et tacot and la
        A TO A COLOR SAN AND A CONTRACT AND A COLOR SAN AND A COLOR SA
        PAAMAEGAOACCTSFAE
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        339732033T9732T8KT0GT0GK8AGGGT0T00GK0GD0GAKAGGCAAAd3 1,050
          3 A 3 V L I V B R L S B A B F N
        GTCAGAGGGTCCTGGGGGGTCGTCGGTGGTTCGGGGGGTCAAGGAGGATGGT 1193
          320700AA30GGGTGTGGGCCCAADGGGGCCTGGCAGGGGGTCAT 1184
          A S M G L S A P M G P S Ç E R V ;
15
        TOGA GCCCARGGCACAGGCACAGGCTGGGCGACCCCATCGAGGCAAAG 1286
          EAHGTSTRIGEPIEA
        20
        NTO POT PAR BTO DATA CETO BECCANO BODO A GLO DO BODO BODO BODO DE LOBO.
          8 1 Y 8 M I G B A Q A A 8 3 M
        FUNCON CANGRISON SON SOCOTOCGGON COGGON COTGCGGCGCACCC () 4 %
25
          TWSCRMSTSARGASCMSTGSCGCREGGCGACGGGGACGGGCGGGGGGCGT 1450
         L H A D E P S P H V D W T A G A V
        CSAADTSSTGACGTCGGSCCGGCCGTGGCCCGAGACCGACCGGCCTAGGS 1500
          ELITSARPWPETDRPE
30
        SGGCAGGCGTGTCGTCCTTCGGGATCAGTGGCACCAACGCCCACGTCATC
       R A G V S S F G I S G T N A H V I
        CTGSARAGOSCACCOCCACTCAGCCTGCGGACRACGCGGTGATCGRGOS (1880
         LESAFPTQFADNAVIER
       GGCACCGGACTGGGTGCCCTTGGTGATTTCGGCCAGGACCCAGTCGGCTT 1650
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         A P E W T F L V I S A R T Q S A
        TGMCTGMGCACCAGGGCCGGTTGCGTGCGTATCTGGCGGCGTCGCCCGGG-1300
        J T D H E G P L R A Y L A A S P G
        BTOCHTHTOGGOGGTTGTGGGATTCGHDGCTGGCGNTGHCACGGTCTTT
         V Q M E A V A S T L A M T F S V F
40
       DOMESA SESTE COGNICO TOGGASK TOACH SECTOACOGS CA COGSTS 1809
          EHRAVILGEBOTVTGTA
       TOTOTGAPPOTOTOGGGGGGTGTTGGTCTTGCCGGGACAGGGGTCGCAGCGT 1889
          STERAMENERGQGSQR
       30T33CAT333TGAGGAAGTGGCCGDCGCGTTCCGCGTCTTCGCGCGAT 1900
45
        A 3 M G E E L A A A F P V F A R 1
       CCATCAGCAGGTGTGGGACCTGCTCGATGTGCCCGATCTGGAGGTGAACG 1950
          H Q I V W S L L D V P D L E V N
       AGACCGCTTACGCCCAGCCGGCCCTGTTCGCAATGCAGGTGGCTCTGTTC
       ETGYAQPALFANQVALE
50
       GGGCTGCTGGARTCGTGGGGTGTACGACCGGACGCGGTGATCGGGCCATTC 1050
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       AMOCOMBURGMANTAGOTST 0050GCGGGCTCGTCTGATGCAGGCTCTGCCC 2150
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       DOGGGTGGGGTGATGGTDGCTGTCCCGGTCTCGGAGGATGAGGCCCGGGC 2200
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       0013073937346337646377677667863666704463667677979366
         V L G E G U E I A A U N G F S S
60
       #86TT0T0T006GT3AT6A36C00C0FT6CTG0A66CCGCAGGGGCGCCCC 2300
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   A R I T D G V A M I B G S B E I
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    CATVERLDIAS V P G F F G
   CCATGSCCGGACGACCGTACAGACCTGGGTCGACGAGCCGGCGGACGACG 3000
   H G R T T V Q T W V D E P A D C
30
   GDOGGOGGOGGTTCACCGTGCACACCGCACCGGGGGACGCCCGGTGGACG 3050
   G R R R F T V H T R T G D A P W T
   OTGCACGCCGAGGGGGTGCTGCGCCCCCATGGCACGGCCCTGCCCGATGO 3200
   L H A E G V L R P H G T A L P D A
   GGCCGACGCCGAGTGGCCCCCACCGGGCGGGTGCCCGGGGACGGGCTGC 3150
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   A D A E W P P P G A V P A D G I
   OGGSTGTGTGGCGCGGGGGGGACCAGGTCTTCGCCGAGGCCGAGGTGGAO 3200
   PSVWRRGDQVFAEAEV
   SGACCGGACGGTTTCGTGGTGCACCCCGACCTGCTCGACGCGGTCTTCTC 3250
   A P S S B V V H P S S S A V F S
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40
   AVGDGSRQPAGWRD1
   TOCACCCCTOGGACCCACCCTACTGCCCCCCTCACCCCGGGCGCACC 2350
   V H A S D A T V L R A C L T P F
   GACGGAGCCATCGGATTOGCCGCCTTCGACGCCGCCGGCCTGCCGGTACT 3400
45
   DGAMGFAAFDGAGLPVL
   CACCGUGGAGGCGGTGACGCTGCGGGAGGTGGCGTCACCGTCCGGCTCCG
    TAEAVTIREVASPSGS
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   GTGTAGGAGGGTGACCTGCCCGAGGGACATGTCCTGATCACCGCCGCCCA 3550
   V Y D G D L P E G H V L I T A A H
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   s v l m a l o e e l m m m d e m l
   ATCGTCCAGACCACCACCGGCCGCCGCCCCCCTCACCGGCCTCAC 3700
   I V H T T T D P A S A T V T G 1 T
   CODERCECTORTOMANACERRORDCCCCRCGCRTTCCCCCTCATCCALACCC 3
    R T A Q M E H P H R I P L I E T
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The AvrII-XhoI restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 13 (specific for methylmalonyl CoA) of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below.

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    TRAPVVPETAATAGÉE
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The *NheII-XhoI* restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 12 (specific for malonyl CoA) of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below.

ASATOTGGGASCTGGCGSAAGCGCTGGTGACGCTCGTGGGGAGAGCACU SI

L A E A L L T L V R E S T

STCSGCGTGTTCGGCCACCTGSGTGGCGAGGACATCCCGGGGACGGCGGC

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F K D L G I D S L T A V C L P V

CCCTCACCGAGGGGGCGGGTGTGGGCTGAACGCCAGGGGGGTCTTCGAC 200

A L T E A T S V R L N A T A V F E

55 TTCCCGACCCGGGAGGTGCTCGCCGGGGAAGGAACTGACCGG 250

F P T F H V L A G K L G D E L T G

CACCGGCGCGCGGTGTCGTCCCCGGGGGGCCACGGCCGGTGGGCACG

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The *Nhe*II-*Nho*I restriction fragment that encodes module 8 of the FK-520 PKS with the endogenous AT domain replaced by the AT domain of module 13 (specific for methylmalonyl CoA) of the rapamycin PKS has the DNA sequence and encodes the amino acid sequence shown below.

	- วิทธานีน มีเกรางว่อเมื่อเมื่อวันเวลาอาจากอนีเวลา เก็บเป็นกรี้	15.
Š	ျားကိုမှုက်၍ ဂန်မျှင်ရှာ နေါ့မှုမေါ့မှုမြော့သို့မြော့ စီမှုခင်မှာ ခန့်နှုပ်မြော့နှင့် ချစ်ချစ်မှု မော်နှင့	0 9 1 • • •
-	TIPO GARANTA ANT ANT ANT ANT ANT ANT ANT ANT ANT	250
	F F T F H V L A G M L G D E L T G DADDOGGEOGGEOGGEOGGEOGGEOGGEOGGEOGGEOGGEOG	300
10	-	350
	GRATTOA DE LA COMENTA DE LA COMENTA DE CARROLA DE CARRO	400
15	CACGGAGTTCCCGACGGACCCCGACCCGACCCGACCCGA	450
	CASACQCSACGCSATOGCCAAGACCTTOGTCCGGCGGGACGGTGCCTTCCTC	500
	ACCIGOCACAGGCTTCCACGGGGCTTCTTGATCAGCCCCCCCGAA	550
20	SUCCOTOGUSATGGACCOSCASCAGOGGGTGCTCCTGGAGACCTCGTGGG A L A M D P Q Q R V L L S T S X	600
	AGBOSTTOGRAAGOODOGGATOROOOOGAOTOGROOGGGGAAAAAC E A F E S A G I T P D S T R G 3 D	630
25	ACCGNIGHTSTHOSHOGGCGCCTTCTCCTACGGTTACGGCACCGGTGCGGA T G V F V G A F S Y G Y G T G A D	700
	CACCGACGCTTCGSCGCGACCGGCTCGCAGACCAGTGTGCTCTCGGGCC T D G F G A T G S Q T S V L S G	750
	GGCTGTCGTACTTCTACGGTCTGSAGGGTCCGGCGGTCACGGTCGACACG	800
30	GCGTGTTCGTCGTCGCTGGTGGCGCTGCACCAGGCCGGGCAGTCGCTGCG A C S S S L V A L H Q A G Q S L R	850
	S G E S L A L V G G V T V M A	900
35	CTCCCGGCGCTTCGTGGAGTTCTCCCGGCAGCGCGCGCCTCGCGCCGGCGGAC	950
	GGCCGGCGAAGGCGTTCGGCGGGGGGGGGGGGGGGGGGG	1000
	- Pagnaceggnangenganegnesasaggenendegabacegaacegaabac - Balaa Balaa Balaa Balaa Balaa Balaa Balaa Balaa	1050
40	STOACACCTOOTGGCGGTCGTCCGTGGTTCGGCGGTCAACCAGAATGST G H T V L A V V R G S A V N Q C G	1100
	GOCTOCAACGGGCTGTCGGCGCGCGGGTGAT A S N G L S A P N G P S Q E R V I	
45	COGSCAGGCCCTGGCCAACGCCGGGGGGGACGTGGACGCCG R Q A L A N A G L T P A D V D A	
	TOGAGGCCACGGCACCGGCACCAGGCTGGGCGACCCCATCGAGGCACAG	
	GOGGTACTGGGCACCTAGGGACAGGAGGGGCCACCCCCTGCTGCTGGG A V L A T Y G Q E R A T P L L L G	
50	S L K S N I G H A Q A A S G V A	
	GOATCATCARGATGGTGCAGGCCTTTTTGGCACGGGGAGCTGCCGCCGACG	1400
55	LHAPEPSPHVGWTAGAV	1450
		1500
	FIGURESCENCE TO STREET TO SECURE STREET SAGE SECOND SECURITIES OF THE FOLLOWING SECURI	
60	OT BBAGGCGGAGCGGTAA DGGAGACGCCGGGGGGTTCGCGTTCCGGTGA	1600

วาที่จากใหม่ที่ระที่ พร้าหาในที่ ขนั้น ผู้ส่วนไทยที่กรกันที่เรียกร้อยอังกับการ จาก (ค.ศ. 2002) (ค.ศ. 2002) (ค.ศ. 2003) (ค.ศ. 2003) (ค.ศ. 2003) 9/100/1900ApAskhaimaappedadaoAoAphineeppeAodobapat 1761 A V A D T L A R R T H F A H R A 10 E L V E V Y S G Q G T 2 H P A M G GAGGAGGTAGGGGTGTTGGGGGAGGGGATGGGGAAGG B Q L A D B S V V F A E R M A E Paggasagerrajagaagrragragacraggarersraadagerrorga 1959 A A A L R E F W D W D L F T V L 15 ACBATCOBGOGGTGB.BBACCGGP.LBATGTCGTCGAGCCCGCTCCCTBB.ECGG D D A V J D R V D V U Ç B A B X 306ATGATGGTTT000T3G00GGGGTGTGGGA3303GG03GTGTGG3300 0050 A M M V S L A A V W Q A A G V E B 20 RARTGCGGTGATGCGGCCATTCGCAGCTGAGATCGCCGAGATGTTGTGTGT daeanacaananakerkosoak<mark>nooccoccoa</mark>ktostakeentacaakaa latea A G A V E L R D A A R I V T : 25 D A I A R G L A G R G A N A S V A CTGCCCCCCAAGGATGTCGAGCTGGTCGACGGGGCCTGGATCGCCGCCC 2250 LPAQDVELVDGAWIAA ACAACGGGCCCGCCTCCACCGTGATCGCGGGCACCCGGAAGCGGTCGAC H N G P A S T V I A G T P E A 7 D 30 CATGTCCTCACCGCTCATGAGGCACAAGGGGTGCGGGTGCGGCGGATCAC H V L T A H E A Q G V R V R R I I CSTOGACTATGCCTCGCACACCCCGCACGTCGAGCTGATCCGCGAGGAAC V D Y A S H T P H V E L I R D E TACTICACATCACTAGCCACAGCAGCTCGCAGACCCCGCTGCTGCCGTGC 2450 35 CTSTCSACCSTSGACGSCACCTGGGTCGACAGCCCSTSGACGGGGAGTA 2500 ls 1 7 pg two bs Plbs er W Y R N L R E P V G F H P A N S 40 AGTT BOAGGOOA GGGGGACACGGTGTTCGTCGAGGTCAGCGCCAGCCCG | 1880 2 A Q G D T V F V E V S A S B 97GTTGTTGCAGGCGATGGACGAC<mark>GATGTCGTCACGGTTGCCA</mark>GGCTGCG 2650 V L L Q A M D D D V V T V A T L A OGTGACGACGGCGACGCCACCGGATGCTCACCGCCCTGGCACAGGCCT 2700 45 R D D G D A T R M L T A L A Q A ATGTCCACGGCGTCACCGTCGACTGGCCCGCCATCCTCGGCACCACCACAA 2750 Y V H G V T V D W P A 1 L G T T T ACCCGGGTACTGGACCTTCCGACCTACGCCTTCCAACACCAGCGGTACTG 2800 TRVLDLPTYAFQEQRYW 50 30TOSAGTOSGCACGCCGGGCGGGATCCGACGGGGGGCACCCCGTGCTGS 2850 L E S A R P A A S D A G H P V L GCTCCGGTATCGCCCTCGCCGGGTCGCCGGGCCGGSTGTTCACGGGTTCC G S G I A L A G S P G F V F T G S 55 V P T G A S A A V F V A B D A L A USCCGGGACGCGGTCGACTGCGCCACGCTCGAGCGCTCGACATCGCCT 3000 A A C A V C C A T V E P L C I A S V P G P P G H G R T T V Q T W V 60 BAGGAGCGGGGGGCGACGGCGGGGGGGGGGGGGGGGGACACCGGGACA

Bartua de como Pullián de brota de toga aste est est estrobroba relicies. VIACTIBOV X B F 3 C ; U F Decama secam et juga no semocam cacarrocar carear central de la Sec ABABY SGPDGFVVBAG TROTOGROSOSCOTOTOTOGOGOTOGOGOAAGOOAAGOOCASCOSGOO 3950 L. L. C. A. V. F. C. A. V. G. D. G. S. F. J. F. A. 10 33A1 290303A 00T3A 09GT3GAOGGGT0GGAOGCA 0QSTACT303030 SW SC DT V H A S D A T V L R A UTG TOTTA DODG SUUDA OGAA OGAA SOCATSG SATTO SOCGOOTTTO SA ON 348 N D L T F R T D G A M G F A A F D 15 SA ATOR OBSTANDSONO SA AGRAGICO SA COSCOTO DA COSCITISTA SIGNISES. A S F S S S B B S D G L H F L E W GOT CECETO CO CARGO CONTACEA CECTO CONTECCO CONT 20 LAVABAVY 9 3 E L 8 E 9 H mobrisk tokność rosej pokopoda zskopodyka skok takopick rozes W L L T A A A B B D D P B D T F F T F GOCCA CA COCCO DOCCACO DOCCOTO COTGACO COT SCAR TA COA CUTUAC - 5700 25 A H T R A T R V L T A L Q H H L I TTDHTLIVHTTTDFAG CCACCGTCACCGGCTCACCCGCACACCGCCAGAACACACCCCCACCGC 3800 ATVTSLTRTAQNEHPHR ATCCCCCTCATCCAAACCCACCCCCACACCCCCCCTCCCCCTCCCCTSGCCCA 3850 30 I R L I E T D H P H T P L P L A Q ACTOGOCACCOTOGACCACCCACCTCCGCCTCACCCACCACCACCACCTCC LATIDHPHLRLTHETL 35 H H P H L T P L H T T T P P T T T COCOTORACCOCGAMCACGCCATCATCATCACCGGCGGCTCCGGCACCCT FLMPEHAIIIOGGSGTL CGCCGGCATCCTCGCCGCGCACCTGAACCACCCCCACACTACCTCCTCT 4050 #FOSTARDECADOS ADSCENCIONADO ACTUADO TOTA TOTA DE ALTOR DE LA TORINO DEL LA TORINO DE LA TORINO DE LA TORINO DE LA TORINO DEL LA TORINO DELLA TRANSPORTIR DEL LA TORINO DELLA TRANSPORTIR DELLA TRANSPORTIR DEL LA TORINO DEL LA TORINO DELLA TRANSPORTIR DELLA 40 GTOGGCGACCCCAACTGGCCACCACCCCCCACACACCCCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCCAACCAACCCAACCCAACCCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACCAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAACAAC LCAIFHTAATLDDGII 45 AGGCCCTCACCCGACCGACCGTCCTCCACCCGAAAGCCAAC 4250 50 V L Y S S A A A V L G S P S Q G ACTACGCOGCGCGAACGCCTTCCTCGACGCCCTCGCCACCCACCCCCAC 4400 U Y A A A N A F L D A L A T H A H ACCCTCGGCCAACCCGCCACCTCCATCGCCTGGGCATGTGGCACACCAC 4450 55 T L S Q P A T S I A W G M W H T T R G G F L F I T D D E G 60

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Phage KC515 DNA was prepared using the procedure described in Genetic Manipulation of *Streptomyces*. A Laboratory Manual, edited by D. Hopwood *et al.* A phage suspension prepared from 10 plates (100 mm) of continent plaques of KC515 on *S. lividans* TK24 generally gave about 3 µg of phage DNA. The DNA was ligated to circularize at the cos site, subsequently digested with restriction enzymes *BamHI* and *PstI*, and dephosphorylated with SAP.

Each module 8 cassette described above was excised with restriction enzymes *BgI*II and *Nsi*I and ligated into the compatible *Bam*HI and *Psi*I sites of KC515 phage DNA prepared as described above. The ligation mixture containing KC515 and various cassettes was transfected into protoplasts of *Streptomyces lividans* TK24 using the procedure described in Genetic Manipulation of *Streptomyces*. A Laboratory Manual edited by D. Hopwood *et al.* and overlaid with TK24 spores. After 16-24 hr. the plaques were restreaked on plates overlaid with TK24 spores. Single plaques were picked and resuspended in 200 µL of nutrient broth. Phage DNA was prepared by the boiling method (Hopwood *et al.*, *supra*). The PCR with primers spanning the left and right boundaries of the recombinant phage was used to verify the correct phage had been isolated. In most cases, at least 80% of the plaques contained the expected insert. To confirm the presence of the resistance marker (thiostrepton), a spot test is used, as described in Lomovskaya *et al.* (1997), in which a plate with spots of phage is overlaid with mixture of spores of TK24 and phiC31 TK24 lysogen. After overnight incubation, the plate is overlaid with antibiotic in soft agar. A working stock is made of all phage containing desired constructs.

Apr 1966, incorporated herein by reference) mycelia were infected with the recombinant phage by mixing the spores and phage (1 x 10⁸ of each), and incubating on R2YE agar (Genetic Manipulation of *Streptomyces*, A Laboratory Manual, edited by D. Hopwood *et al.*) at 30°C for 10 days. Recombinant clones were selected and plated on minimal medium containing thiostrepton (50 μg/ml) to select for the thiostrepton resistance-conferring gene. Primary thiostrepton resistant clones were isolated and purified through a second round of single colony isolation, as necessary. To obtain thiostrepton-sensitive revertants that underwent a second recombination event to evict the phage genome, primary recombinants were propagated in liquid media for two to three days in the absence of thiostrepton and then spread on agar medium without thiostrepton to obtain spores. Spores were plated to obtain about 50 colonies per plate, and thiostrepton sensitive colonies were identified by

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replica plating onto thiostrepton containing agar medium. The PCR was used to determine which of the thiostrepton sensitive colonies reverted to the wild type (reversal of the initial integration event), and which contain the desired AT swap at module 8 in the ATCC 14891-derived cells. The PCR primers used amplified either the KS AT junction or the AT/DH junction of the wild-type and the desired recombinant strains. Fermentation of the recombinant strains, followed by isolation of the metabolites and analysis by LCMS, and NMR is used to characterize the novel polyketide compounds.

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Example 2

Replacement of Methoxyl with Hydrogen or Methyl at C-13 of FK-506

The present invention also provides the 13-desmethoxy derivatives of FK-506 and the novel PKS enzymes that produce them. A variety of *Streptomyces* strains that produce FK-506 are known in the art, including *S. tsukubaensis* No. 9993 (FERM BP-927), described in U.S. Patent No. 5,624,852, incorporated herein by reference: *S. hygroscopicus* subsp. *yakushimaensis* No. 7238, described in U.S. patent No. 4,894,366, incorporated herein by reference; *S.* sp. MA6858 (ATCC 55098), described in U.S. Patent Nos. 5,116,756, incorporated herein by reference; and *S.* sp. MA 6548, described in Motamedi *et al.*, 1998, "The biosynthetic gene cluster for the macrolactone ring of the immunosuppressant FK-506," *Eur. J. Biochem. 256*: 528-534, and Motamedi *et al.*, 1997, "Structural organization of a multifunctional polyketide synthase involved in the biosynthesis of the macrolide immunosuppressant FK-506." *Eur. J. Biochem. 244*: 74-80, each of which is incorporated herein by reference.

The complete sequence of the FK-506 gene cluster from *Streptomyces* sp. MA6548 is known, and the sequences of the corresponding gene clusters from other FK-506-producing organisms is highly homologous thereto. The novel FK-506 recombinant gene clusters of the present invention differ from the naturally occurring gene clusters in that the AT domain of module 8 of the naturally occurring PKSs is replaced by an AT domain specific for malonyl CoA or methylmalonyl CoA. These AT domain replacements are made at the DNA level, following the methodology described in Example 1.

The naturally occurring module 8 sequence for the MA6548 strain is shown below, followed by the illustrative hybrid module 8 sequences for the MA6548 strains.

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GUNTGOGGOTOTACGAGGOGGAAGGGGGAAGGGGGAAGTCCCGTGGTGGTG 50

M.R. L. Y. E. A. A. R. R. T. G. S. P. V. V. V.

GCGGCCGGGGTUGACGAGGGGGCGCGGGACGTGCCGGCGGGGCTGCG
A. A. A. L. D. D. A. F. D. V. F. L. L. P. G. L. A.
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RAAAVEEBSLAS
                      ????3393432523433???<mark>????#63440?0038</mark>4?0380?020798993393333
             FAITTERELSICSITA
           TCOAGOTTOGUSÁA DAGOTTOÁG TÁGGGGGÁGGGGGGTTAGGGGTTTÁA DUGG
TOUR LONG HOR AND TOUR ANT GOVER LOUGA
10
           NGA GAA GOT GBODGGTAK COBCBOGCC GOT GGC BBCCCGGBAACCBC BBCCA - 45.0
             D E L A G T R A F V A A R T A A
15
           $ 000 TOCCCTTSCTASCCTCCTTGGGGATGGGGAAGGGACACGCCCTGGGGAT
            AAAHBEPLAIVIMA
           TTGCCGGGGGGGGGGGCGTCGCCACAGGAGCTGTGGCGTCTCGTGGCGTC
            L P G G V A S P Q E L W R L V A S
           CGOCACCAR CGCCATCACCGAGTTCCCCCGGACCGGGGTGGGACCGGACCGGA
20
             G T D A I T E P P A D E G W D V
          A 339GCTETA COM COORDA COORDA COORATO SCARA A COTTO STOCKE 650
            NA DOGO OGOTT COM DGA COGREGO<mark>AN COGORMACA A COCOCOMMETT</mark> COG
           H G G F L D G A T G F C A A F F G
25
          GMTCAGCCCGCGCGAGGCCCTGGCCATGGACCGCAAGCAAACGCCTCC
            DOS DUBORDOTOTTO GOLAGO STROGRAMO DO GOLA CONTREBENTO DO CONTREBENTO DE CONTREBENTA DE CONTREBEN
            RTSWEAFESAGITFOA
          BOGGGGGGGAGGGACACGGGGGGGGTTTCATGGGGGGGTTCTCCTACGGGTA 850
30
           ARGSDTGVFIGAFSYGY
          CGSCACGGGTGCGGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA 900
            STGADTNGFGATGSQT
          GOGTGOTCTCCGGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950
          S V L S G R L S Y F Y G L E G F S
35
          STCACGGTCGACACCGCCTCCTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000
           V T V D T A C S S S L V A L H Q A
          AGGGCAGTCCCTGCGCTCGGSCGAATGCTCGCTCGCCCTGGTCGGCGGTG 1050
            3 9 8 1 8 8 3 E C 8 L A 1 7 3 3
          TONO SCHOME GGOGT COCETAGGGGGATHOCTOCA GTHOTOCCGGGAGGGGG-1100
40
          T T N-M A S P G G-F V E F S R 2 R
          30307<mark>0</mark>30450334,0336033303AAGGGSTT0363326G633333AGG8-1156
           G L A F C G A A K A F G A G A D G
          TACGAGOTTOGOCGAGOGOCCGGTGCCCTGGTGGTCGAGCGGCTCTCCG 1200
            TSFAEGASALVVERLS
          ACCCGGASCGCCACGGCCACACCGTCCTCGCCCCTCGTACGCGGCTCCGCG 1250
45
          DARREGETVLALVROSA
          SCTAACTCCGACGGGGGGTCGAACGGTCTSTCGGCGGCGAACGGCCCCTC 1300
           ANSDGASIGLSAPNGPS
          CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCCG 1350
50
           QERVIHQALANAKLTP
          COGATGTOGACGOGGTOGAGGOGCACGGCACCGGCACCGCCTCGGCGAC 1400
          A D V D A V E A H G T G T R L G D
          SCONTOGREGOGORGEGOGOTGOTOGOGACGTROGGROAGGROOGGGOGRO 1450
           PIEAQALLATYSQERA:
55
          SCCCCTGCTGCTCGGCTCGCTGAAGTCGAACATCGGGCACGCCCAGGCCG 1500
             PLLLGSLKSNIGHAQA
           OTCAGGGGTOGCGGGATCATCAAGATGGTGCAGGCCATCCGGCACGGG 1550
          A S G V A G I I K M V Q A I B B G
          GAACTGCCGCCGACACTCCACCCGGACGAGCCGTCGCCGCACGTCGACTG 1600
60
           ELPETLHADEFSPHVDW
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	- RAMBODUQUTGOUNTIGABUTTATMAAQSTOGGOUTGOUTGOUGGGGGAA - TO A GAN WEELD DO TOO A BURNEY OF B	1850
	TO TALIBURGE BE BE BE BET DU COMUNIO PROBET, NE BETÜR BABEBBARB.	1-7:
5	- AA SBODDADAT BÂN TOTT BABBABBABBBT TÂ AAAÂ SBBBANT BODD ÂA. DE ABABBABBAT BABBABBABBABBABBABBABBBABBBA	1750
	- RBCABBARCBATBBARBCARBBARCBBTBBARBTABARCBBBT	1400
10	GACTOSTOCCOSTOSTOCCOSTORACO SE SA ASACUTTO SOCTO	1850
	- CTCGTGTGGGGGGGTTCGGGGGGGGGGGGGGGGGGGGG	1900
	BIGGGGGTATICTCGACACTGGGCGGGGGGGGGGGGGGGGG	1950
15	AGACACTGGCCGGGGTACGCACTTCACCCACCGGGGCGTACTGCTCGGG	2000
	GARRICOGTOMT GGOGOTOCILOCGGGGGGGGGGGGGGGGGGGAGGAAGTGGTCTT L T V I G A P P A D Q A D E I V F	2050
20	COTOTACTOCOCTCAGOCACCAGCATCCCCGCATGGGCGAGCAACTCCC	2100
	CBBCCBCGTTCCCCGTGTCCBCGATGCCTGSCACGACGACGCGTCCGACGG	2150
	STOSACGACCCCACCCCCCCCCCCCCCCCCCCCCCCCCCCCC	2200
25	OSCOCACCAGOCOCOTCACCOCOCOCOCAGOCOCOTOGGACATOACGC	2250
	•	2300
30	GODDAGATECTGTCGCTCGACGACGCCTGCACCCTGATCACCACGCGTGC	2350
	EGGCCTCATGCACACGCTTCCSCCGCCCGGCGCCATGGTCACCGTGCTGA E. L M H T L F P P G A M V T V L	2400
	8. L M H T L F P P G A M V T V L COASCGAGGAGGAGGCCCGTCAGGCGCTGGGGCCGGGGCGTGGAGATCGCC	2400 2450
35	EL M H T L E P P G A M V T V L CCASCGAGGAGGAGGCCCGTCAGGCGCTGGGGCCGGGCGTGGAGATCGCCT S E E E A R Q A L R P G V E I A SCGGTCTTCGGGGCGAGGACGAGGACGCGT	
	ELM H T L F P P G A M V T V L CCASCGAGGAGGGCCCSTCAGGCGCTGGGGCCGGGGCGGGG	2450
	ELM H T L F P P G A M V T V L CCASCGAGGAGGCCCGTCAGGCGCTGGGGCCGTGGAGATCGCC T S E E E A R Q A L R P G V E I A SUGSTCTTCGGGCGCGAGGAGGACGCGT A V F G P H S V V L S G D E E A V GCTCGACGACGACGACGACGACGACGACGACGACGACGACGAC	2450 2500
35	ELM HIT LE PPG AMVIT VL CCASCGAGGAGGAGGCCGTCAGGCGCTGGGGCCGGGGGGGGGG	2450 2500 2550 2600
35	ELM HIT LEPPGAM VIT VL CCASCGAGGAGGAGGCCGGTCAGGCGCTGGGGCCGTGGAGATCGCC TSEELARQA LERPGVEIA SUGSTCTTOGGCCGCCACTCCGTGCTCTCGGGGCGAGAGGAGGCGGT AVFGPHSVVLBSGCACGAGGAGGAGGAGGCGGC LEVAQER LGIRHFLFLFAP ACCCGCCCACTCCGCCCACCACCACCGCCGAGCTCTGCCCGCCC	2450 2500 2550 2600 2650
35	ELM HIT LEPPGAM VIT VL CCASCGAGGAGGAGGCCGTCAGGCGCTGGGGCCGTGGAGATCGCC TSEELEAR QALBRPGVEIA SUGSTCTTOGGCCGCACTCCSTCGTGCTCTCGGGGCGAGAGGAGGCGGT AVFGPHSVVLSSGCATCCGCGAGGAGGAGGAGGAGGCGT AVFGPHSVVLSSGCATCCACCACGACGGCCGCCCCCCCCCCCCCCCCCCC	2450 2500 2550 2600 2650 2700
35	ELLM HIT LEPPG AM VIT VL CCASCGAGGAGGAGGCCCTCAGGCGCTGGGGCCGGGGGTGAGATCGCC TSEES ARQALLRPG VEIA SCGGGTTTTGGGCCGGACTCCGTGGTGTTTCGGGGGAGAGGAGGAGGCGTT AVFGPPHSVVLLSGCTTTGGGGGAGAGGAGGAGGCGT AVFGPPHSVVLLSGCTTTGGGGGGAGGAGGAGGCGT AVFGPPHSVVLLSGCTTTGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG	2450 2500 2550 2600 2650 2700 2750
35 40 45	ELLM HIT LEPPG AM VIT VL CCASCGAGGAGGAGGCCCTCAGGCGCTGGGGCCGGGGTGGAGATCGCC TSEES ARQALLRPG VEIA SCGTTTTGGGCCGCACTCCGTGGTGTGTGTGGGGCGGGGGGGG	2450 2500 2550 2600 2650 2700 2750 2800
35	ELLM HIT LEPPG AM VIT VL CCASCGAGGAGGAGGCCCTCAGGCGCTGGGGCCGGGGTGGAGATCGCC TSEES ARQALLRPG VEIA SCGTTTTGGCCCGCACTCGTGTGTGTGTGGGCGGGGGGGGG	2450 2500 2550 2650 2700 2750 2800 2850
35 40 45	EL M H T L F P P G A M V T V L CCASCGAGAGAGGCCCSTCAGGCGCTGCGGCCGGGCGTGAGATCGCC T S E E E A R Q A L R P G V E I A SUSTITTOGSCCGGACTCCSTCGTGTGTGTGGGCAGAGGAGGAGGCGGT A V F G P H S V V L S S D E E A V SCTCSACGTCGCACAGGGGTTGGGATCCACCACGGTTTGGGGGCGGGC	2450 2500 2550 2650 2700 2750 2850 2900
35 40 45	E. L. M. H. T. L. F. P. P. G. A. M. V. T. V. L. CCASCGAGGAGGGCCCTCAGGGGCCGGGCCGGGCGTGGAGATCGCC T. S. E. E. A. R. Q. A. L. R. P. G. V. E. I. A. SUGSTICTTOGGGCGGGCGGGGGGGGGGGGGGGGGGGGGGGGGG	2450 2500 2550 2600 2650 2750 2850 2850 2950
35 40 45	ELMHHTLEFPPGAMVTVL CCASCGAGGAGGCCCGTCAGGCCGTGGGCCGGGCGGGGAGATCGCC TSEEEARQALABRY VEIA SCGSTCTTCGGCCCGCACTCCGTCGTGCTCTCGGGCCGGAGGACGCGGT AVFGPHSVVLBSGDEEAAGGGCGGGCGGCGGCGGT AVFGPHSVVLBSGDEEAAACGCGTCTGCGGGCGGGCGGCGCGCCLDVAAQACCCGCGCGCGCGCCACACGGGCGGGCGGCGCGCCACACGGGGCGGGCGGGCGGGCGGGCGGGGGG	2450 2500 2550 2650 2700 2750 2850 2900 2950
35 40 45	ELMBET LEPPGAMVT VLCCASCGAGGAGGAGGAGGCCGTCAGGCCGTGGGCCGGGCC	2450 2500 2550 2650 2700 2750 2850 2850 2900 3050

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- Barrier Barrier (n. 1906).
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NGA DIGONTO GT GGA OGOT GCA OGOGGA GGG GOTTOTION I DIGONNA UDGGGG (1930).
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               RE DESTROCCER RESERVATA CENTROCA CON CONTROL A CONTROL A CONTROL A CONTROL CON
25
                   PVABAHYDGALB1PEG
               30
                 PHNTPTRTHTQTTRVLS
               ALCHHLITTNETLIVE
               T T T D P P G A A V T G L T F T A
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               HAILITGGSSGTLAGIL
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45
               A R H L N H P H T Y L L S R T P P
               CCAMATOMOUGAAGOCCTOACCOMOATACOACMACCCCTCACCGGCATCT 4350
50
                  QITQALTHIPQPLTGI
               TOUNCACOGOGGCACCCTGGACGACGCCACCCTCACCAACCTCACCCCC 4400
               F H T A A T L D D A T L T P
               CAACACCTCACCACCACCCTCCAACCCAARGCCGACGCCGCCTGGCACCT 4450
                 Q H L T T T L Q P K A D A A W H L
55
                 CACCACCACACACAAAACCAACCCCTCACCCACTTCGTCCTCTACTCCA 4500
                  H H H T Q N Q P L T H F V L Y S
               GOGCCGCCGCCACCCTCGGCAGCCCCGGCCAAGCCAACTACGCCGCCGCCC 4550
               S A A A T L G S P G Q A N Y A A A
              AACGCCTTCCTCGACGCCCTCGCCACCCACCCCCACCCCAAGGACAACC 4600
60
               NAFLDALATHRHTQGQP
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5

The AvrII-Xhol hybrid FK-506 PKS module 8 containing the AT domain of module 12 of rapamycin is shown below.

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10
        30ATG0g60T6TAC$A66C6GAACG6G$AACG7AAAGTCCCTCCCCCCCCCC
             M F 1 Y E A A F F T G 3 P V V
         A A A L D D A F D Y P L L & G L R
        FORTA CAN CORTOGOGOSTA TARACETTOGOGORA CO CTOTOTOGOGORA CO 150
           B T T V B P A A V B E R S L A D
        GETEGRESTSCTGSCCGMCGASGAGGGGGGGGAEGGCTSSCTCGSCGTTDG 200
        R S P C C P T T S A P T P P S R S
        TOOTGGAACAGCACCGCACCGTGCTCGGCCACCTGGGCGCGAAGACAT 250
          S W N S T A T V L G H L G A E D I
20
        COCGCCACCACGACGACGAAGGAACTCGGCATCGACTCGCTCACCCCC
          FATTFHELGIDSLTA
        TOCAGOTGOGGAACGCGGTGACCACGACGACGATAGGCCTCAACGCCT
          QLANALITÀTGVELNA
        ACAGOGOTETTOGACTTTOGGACGCGCGCGCGCGCGCGAGACCTCGG 400
25
        TAVEDEPTERALAARIG
        GGACGAGCTGGCCGGTACCCGCGCGCGCCCGGCCCGGACCGCGCCA 450
           D E L A G T R A P V A A R T A A
        DOGGGGGGGGGACGACGAACCGCTGGGGATCGTGGGGCATGGCCTGCCGT 500
        TAAAHDEFLAIVGMACR
30
        CTGCCGGGGGGGGTCGCCTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC 550
        L P G G V A S P Q E L W R L V A S
        OGSCACCGACGCCATCACGGASTTCCCCGCGACCGCGGCTGGGACGTGG 600
            \texttt{G} \ \texttt{T} \ \texttt{D} \ \texttt{A} \ \texttt{I} \ \texttt{T} \ \texttt{E} \ \texttt{F} \ \texttt{P} \ \texttt{A} \ \texttt{D} \ \texttt{R} \ \texttt{G} \ \texttt{W} \ \texttt{D} \ \texttt{V}    
        ACGCGCTCTACGACCCGGACCCGACGCGATCGGCAAGACCTTCGTCCGG 650
35
        DALYDPDFDAIGKTFVR
        CACGGCGGCTTCCTCGACGGTGCGACCGGCTTCGACGCGGCGTTCTTCGG 700
        H G G F L D G A T 3 F D A A F F 3
        GAT CAGGCCGCGCGAGGCCCTGGCCATGGACCGGCAGCAACGGGTGCTCC 750
         I S P R E A L A M D P Q Q R V L
        TGGAGAGGTCCTGGGAGGCGTTCGAAAGCGCGGGCATCACCCCGGACGCG 800
40
        LETSWEAFESAGITPDA
        GCGCGGGGCAGCGACACCGGCGTGTTCATCGGCGCGTTCTCCTACGGGTA 850
        A B G S D T G V F I G A F S Y G Y
        DUGCACGGGTGCGGATACCAACGGCTTCGGCGCGACAGGGTCGCAGACCA 900
45
        STSADTNGFGATGSQT
        GCGTGC.CTCCGGCCGCCTCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950
        S V L S G R L S Y F Y G L E G P S
        STOACGGTCGACACCGCCTGSTCGTCGTCACTGGTCGCCCTGCACGAGGC 1900
        V T V D T A C S S S L V A L R Q A
5Û
       AGGGCAGTCCCTGCGCTCGGGCGAATGCTCGCTCGCCCTGGTCGGCGGGTG 1050
        G Q S L R S G E C S L A L V G G
        TCACGGTGATGGCGTCGCCCGGCGGCGCATTCGTCGAGTTCTCCCGGCAGCGC 1100
        V T V M A S P G G F V E F S R Q B
        55
        G L A P D G R A K A F G A G A D G
        TA OGR O TITO SO CARGO COSO SO SO CORO SO CARGO CONTIDO CARGO CARGO CONTIDO CARGO CA
         T S F A E G A G A L V V E R L S
        ACGCGGAGCGCCACGCCCACACCETCCTCGCCCTCGTACGCGGCTCCGCG 1250
        D A E R H G H T V L A L V R G S A
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Cawasie o Bharakhalaka na Baddanasasie a Badase na badasi 1380
                                                                    TABLE 1
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 10
           GAMOTGOUGOUGÁCACTSCACGOGGACGAGCCGTOGCGGAGACTUMAUNG 1600
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           CASAVELETSAREWES
               CORDECTEROTRESSOPPODOPOPOROSORAGESOSSATECCOSTEC
               NABVILESAPFOQPASM
 20
           082 FUTURE TO RECEDENCE DE CONTRECE DE CONTRECE CONTRECE CONTRE CONTRECE CO
               A V I E B A F E W V F L V I
           GBADDOAGTOGGCTTTCACTGAGGAGGAGGGGGGTTGGGTGDGTATGTG 1889
           FTQSAITEHEGPLRATI
           SCSSSSTCGCCCGGGGTGGATATGCGGGCTGTSGCATCGACGCTSSCSAT 1903
             A A S P G V D M R A V A S T L A M
           SACACGGTCGGTGTTCGAGCACCGTGCCGTGCTGCTGGGAGATGACACCG 1950
                 REVEEHRAVILGOOT
           TOACOSCACOGOTOTOTOACCOTOGGGGGTGTTCGTCTTCCCGGGA. 8000
30
           V T G T A V S D P R A V F V F P G
           CAGGGGTCGCABCGTGCTGGCATGGGTGAGGAACTGBCCGCCGCGBCGTTCCC 2050
             J G S Q R A G M G E E L A A A F P
            OFFITTOGOGGGGATCCATCAGCAGGTGTGGGACCTGCTCGATGTGCCG
              V F A R I H Q Q V W D L L D V P
          ATOTAGA GGTGALAGGA GA GGGGTTA GGGGGCAGCGGGGGGGTGGT, GGGA ATG 2153
35
          E L E V N E T G Y A Q P A L F A M
           CAGGTEGOT STETTOESSOCTGOTGGAATOGTGGGGTGTACGACCGGACGC 2200
             I V A L FIG L L B S W G V B F D A
           3010A POGGOORTTOGGTGAGGTTGGGGGGTGTGTATGTGTDDGGGG 2001
40
             V 1 G H S V G E L A A A Y V S 3
          TSTGGTCGTT3GAGGATGCGTGCACTTTGGTGTCGGC3GCGGGCTCGTCTG 2350
           V W S L E D A C T L V S A R A R l
          ATGCAGGGTCTGCCCGCGGGGTGGGGGTGATGGTCGCTGTCCCGGTCTCGGA 2350
           M Q A L P A G G V M V A V P V S E
45
          GGATGAGGCCCGGGCCGTGCTGGGTGAGGGTGTGGAGATCGCCGCGCGTCA 2400
               S E A R A V L G E G V E I A A V
          ACGSCHOGTOGTOGGTGGTTCTCTCCGGGTGATGAGGCILLUGTGCTGCAG 2450
          H G P S S V V L S G D E A A V L Q
           3009003AGG63dT696GAMGT66ACGCGGCGT6GCGACCAGCCACCCGTT 2500
50
            AAEGLGKWTRLATSHAF
           CCATTOCGCCCGTATGGAACCCATGCTGGAGGAGTTCCGGGGCGGTCGCCG-2550
              H S A P M E F M L E E F P A U A
          AAGGCCTGACCTACCGGACGCCGAGGTCTCCATGGCCGTTGGTGATCAG | 2600
          B G L T Y B T P Q V S M A V G C 2
55
          GFGACCACCCCTGAGTACTGGGTGCGGCAGGTCCGGGACACGGTCCGGTT 2650
           V T T A E Y W V R Q V R D T V R E
           DGGCGAGCAGGTGGCCTCGTACGAGGACGCCGTGTTCGTCGAGCTGGGTG 2700
              BIVASTEDAVECUS
          CCGACCGGTCACTGGCCCGCCTGGTCGACGGTGTCGCCGATGCTGCACGGC 2750
60
          A D R S L A R L V D G V A M L H G
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ACCAL AMOTORIAGED AND COCCOCOTANCE CONTACTOR ACTOR ACCOTANAL CAROLA THE ET LAAT GALABLY V.N. GGTGGKCTGGGGGGGGGGTCGTGGGGGAKTGGTTGGGGAAACAG 2850 i m i u p w F A L L P i A F A T gaga yan asabanta nakakakan mada mada sakan asaban 2800 THE AND A GOOD FOR MIT SOF 10 FAGACEAVELAELALAE Bodarosoorobarotadaoorr<mark>ogo</mark>osrmorsoodarestordategat (3107 A C A T T O A T W E L L T W T S W TO S W TO SO 15 Pasal Asak Alak Ja AMBORGOUGOBA OGGGGGGGGGGGGTT DA OGGT GCA CA COCGGGT DAYA (1920) GAOGOODOGTGGAOGOTGUACGCCGAGGGGGTTCTCCGGCCCCGGCCGCGT 3250 DABWTLHAEGVLRP3RV 20 PIPEAUDTAWFPPGA PADGLPGAWRRADQUF SAAGCCSAAGTCSACAGCCT<mark>GACGGCTTCGTG</mark>GCACACCCCGACCTGCT-3400 25 E A E V S S P D G F V A B P D L DGA DG DG TOTTOTOG SOG TOG GOG ACCGAGO SCOAGO SACCGAT 3450 C A V F S A V G D G S R Q P T 3 DECEMBER OF THE PROPERTY OF TH W P I L A V H A S D A T V L R A C 30 CTCACCCCCCCACAGTGCTGTGTGGAGCTCGCCGCCTTCGACGGTGC 3550 L T R R D S G V V E L A A F D S A CGGAATGCCSGTGCTCACCGCGGGAGTCGGTGACGCTGGGCGAGGTCGCGT 3600 G M E V L T A E S V T L G E V A DGGCAGGCGGATCCGACGAGTCGGACGGTCTGCTTCGGCTTGAGTGGTTG 3650 35 SASSSESDGLLRLEW. 00F6 ATTREEPARPOORTSACEADORDOORTSACEATRACERSCORRESSATA 00F6 E M A E A H M D G A C E L P E G M CACUCTCATCACCECCACACACCCCGACGACCCGACGACCCCACCAACC 3750 40 CCCACACACACACCCACACCCACACACACACACACACGCGTCCTCACC 3800 PHHTPTRTHTQTTRVLT GCCCTCCAACACCACCATCACCACCACCACCACCACCATCGTCCACAC 3850 ALQHELITTNHTLIVHT 45 TTTPPPGAAVTGLTRTA ACCCCACTOCOCCTCACTCACTCACCACCCTCCACCAACCCCACCTACG 4000 50 T F L F L T 2 L T T L H Q F H L R ACCACACACACCACCACACACCCCCAACACCCCCACCCCTCAACCCCAAC 4100 H H H T T T T T P N T E P L M P N 55 CARGOCAT COTOATCACOGGOGGCTCCGGCACCCTCGCCGGCATCCTCGC 4150 RAILITGGSGTLAGILA COGCONCOTORACOMOCOCORSACCTACCTCCTCTCCCGCACACCACCAC RELWEFETYLLSRTF COCCACACACCCGGCACCCACATCCCCTGCGACCTCACCGACCCCACC 4250 PPT PST HIPCDLT DPT 60

The AvrII-AhoI hybrid FK-506 PKS module 8 containing the AT domain of module 13 of rapamycin is shown below.

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GCATGCCCTGTACGAGGCCGCAACGGCGCACGGAAAGTCCCCGTGGTGGTG
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         A A A L E D A P D V P L L R G L R
         GOGTACGMCCOTTCCGGCGTGCCGCCGTCCGGGGAACGCTCTCTCGCCGACC 150
           STTVRRAAVRERSLAD
         GOTOGOGTGOTGOCCGACGACGACGCGCGCGCGCCTCCCTCGCGTTCG 200
         RSPCCPTTSAPTPPSRS
         TOCTUBAACAGRACOGOCACOGTGCTCGGCCACCTGGGCGCGAAGACAT 250
          SWNSTATVLGSLGAECI
         SCCGGCTACGACGACGTTCAAGGAACTCGGCATCGACTCGCTCACCGCGG-300
           FATTFRELGIDSLTA
         TOURSCROPSCAR DEGGETGROCK CEGGGGACCGGGGTRACGGCTCARCGCC - 350
35
           QLESALTTATGVELNA
         A0A30397177703A37777033A0900303030303070380303A3A370330 488
           SAUSOBPTSRALAARIG
         DELAGTRAPVAARTAA
40
         COGCCCCCCACGACGAACCCCTGCCGATCGTGGGCATGGCCTGCCGT 500
        T A A A H D E P L A I V G M A C R
         CTGCCGGGGGGGTCGCGTCGCCACAGJAGCTGTGGCGTCTCGTCGCGTC 550
          L P S S V A S P Q E L W R L V A S
        CGGCACCGACGCATCACGGAGTTCCCCGCGGACCGGCGGCTGGGACCTGG 600
45
          G T D A I T E F P A D R G W D V
        ACGCSCTCTACSACCCGGACCCCGACGCGATCGGCAAGACCTTCGTCCGG 650
         DALYDPDPDAIGKTFVR
         CACGGCGGCTTCCTTCGACGGTGCGACGGCGCTTCGACGCGGGGTTTCTTCGG 700
         H G G E L D G A T G F D A A F F G
50
        GATCAGCCGGGGAGGCCGTGGCGATGGACCGCACCAAGGGGTGCTCC 750
          TAGAGAGGTCCTGGGAGGGGTTGGAAAAGGGGGGCATCATCCGGGAGAGGGG 800
         LECSWEAFESAGITEDA
        SOSCOUSSEACACOGOGGTSTTCATCGSCSCGTTCCCCTACGSGTA 850
55
         A S S S D T G T F I G A F S Y G Y
         COSCA UBBBT BEBGATACOAR OBBCTTOCGGBABACABBGTCGGABACCA | BCC
           G T G A D T N G F G A T G S Q T
         SOGTGOTOTOOSGOOGGOTOTOGTACTTOTACGGTCTGGAGGGCCCTTCG 950
         SVISGRISYFYGLEGPS
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 25
                E L P P T L H A D E P S P H T E W
               GACGGCCGCTCCAGCTCCACGTCGGCCCGGCCGTGCCCGGGGAA 1859
                   TAGAVELLTSARPWF3
                COUGTOGCCCTAGGCGGGCGGGCGTGTCGTCCTTCGGAGTCAGCGGCACC
 30
               T G R P R R A G V S S F G V S G T
               AACGCCCACGTCATCCTGGAGAGCGCACCCCCCCCCTCAGCCCGCGGGAAGGA 1750
                NAHVILESAPPAQPAEE
               SGCGCAGCCTGTTGAGACGCCGGTGGTGGCCTCGGATGTGCTGCCGCTGG 1800
                    AQPVETPVVASDVLF
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               TOATATOGOCCAAGACCCAGCCCGCCTGACCGAACACGAAGACCGGCTG 1850
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                               TACOTGGGGGGTGTGGGGGGGGGGGGGGGGATATAGGGGGCTGTGGGGATGT 1900
                FAYLAASPGATIAAVAS
               SACCOMBOGGOMOROGGTOGGTOTTTGROCACCACCCCCAROTTCCTTCC 1986
40
                  I LAVTRS V PEHRAV 1
               GAGATGACACOGTCACCOGOCACCOGGGTGACCGACCAGGATCGTGTTT 2000
               3 D D T V T G T A V T D P R I V F
               STOTTTOOCGGGCAGGGGTGGCAGTGGCTGGGGATGGGCAGTGCACTGCG-2050
                 V F P G Q G W Q W L G M G S A L R
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               CGATTCGTCGGTGGTGTTCGCCGAGCGGATGGCCGAGTGTGCGGCGGGGGGT 2100
                  ESSVVFAERMAECAAA
              TGGGGGAGTTGGTGGACTGGGATCTGTTGACGGTTCTGGATGATGGGGGG
                 . REFVOWDLFTVLDDPA
               STORTGRACCGGGTTGATGTGGTCCAGCCCGCTTCCTGGGCGATGATGGT 2200
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               TOBSOCATTOGCAGGGTGAGATCGCCGCAGCTTGTGTGGCGGGTGGGGTG 2300
                   SHSQGEIAAACVASAV
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               TORPTROOPSHTGOOGCOGGATOSTGROOTTSDSCRGOCAGGGANDESS (235)
                S L R D A A R I V T L R S Q A I A
               07393600T6306650C3666C3C3AMG3CAMD03T6G00CMGCC3C3C
                   3 3 1 A G E G A M A S I A L P A
              AGGATGTCGAGCTGGTCGACGGGGCCTGGATCGCCGCCACAACGGGCCC 2450
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           GOTTEVEVSASEV
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        GOGALISCA CONGGATGOTICA DOGCOOTSGOA CAGGOOTATGTOCACGIO 2960
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       TGDGGACCGCGCGCTGTTCATCGCCGAACTGGCGCTCGCCGCCGCCGACG 3150
          A D R A V F I A E L A L A A A D
       CCACCGACTGCGCCACGGTCGAACAGCTCGACGTCACCTCCGTGCCCGGC 3200
       A T D C A T V E Q L D V T S V P G
30
       GGATCCGCCGGGGAAGGGCCACCGGGGAGCCTGGGTGGATGAACCGGC 3250
        G S A R G R A T A Q T W V D E P A
       CGCCGACGGGGGGGGCCCGCTTCACCGTCCACACCCGCGTCGGGGACGCCC 3300
          A D G R R R F T V H T R V G D A
       CTTGGACGCTGCACGCGAGGGGGTTCTCCGCCCCGGGCGCGTGCCCAG 3350
       PWTLHAEGVLRPGRVPQ
          odgaagoogtogaoacogtofggddooggggggggggggggggggga 3400
         FEAVOTAMPPPGAVPAC
        agasambaaagagaagmaaagaaaaaagaaaganaangamammagaasanaasas 3450
       3 L 9 3 A W R R A D D V F W E A AGETOGRADADECTRATIGACECE 3500
40
       E V D S F D G F V A H P D L L D A GTOTTOTOGGGGTGGGGGAGGGGAGGGCAGGGGAGGGGATGGGGGA 3550
        V F S A V G D G S R Q P T G W R D
        45
         LAVHASDATVLRACLT
       GUOGOGACAGTGGTGGTGGAGCTCGCCGCUTTCGALLGTGCCGGAATG 3650
       R R D S G V V E L A A F C G A G M
       CCGGTGCTCACCGGGGAGTCGGTGACGCTGGGGAGGTCGCGTCGGCAGG 3700
       FVLTAESVTLGEVASAG
50
       CBGATCCGACGAGTCGGACGGTCTGCTTCGGCTTGAGTGGTTGCCGGTGG 3750
          G S D E S D G L L R L E W L P A
       CGGAGGCCACTACGACGGTGCCGACGAGCTGCCCGAGGGCTACACCCTC 3800
       A E A H Y D G A D E L F E G Y T L
       ATOROGOOMOROMOODOGAOGAOCOGAOGAOOODAOCOGOADAR 3850
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        ON CAMBOR ON COOR DOCK CAMANDEADAN ACONTROS COTOROS COMOS 1900
            ратавт но оттальтал
       AACACCROSTOATCACCACCAACCACACCCTGATGGTGCACACCACCACCACC
       60
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   LHPHTYLLSATPPPPT
   DA CACCOGGO A COCA CATOCCOTGO GACCTCACO GACCO DA COCAAAT DA 4350
    TPGTHIPCDLTDPT
   OCCAAGOOCTCAOCCACACACACACCCCTCACOGGCATCTTCCACACC
   TOALTHIPOPLIGIFET
   20
   CACCACCACCCTCCAACCCMMSCCGACGCCCTGGCACCTCCACCACC
    T T T L Q F K A D A A K H L H H
   ACACCOAAAACCASCOCTCACCOMOTTCGTCCTCCTCCAGCSCSCSCC 4550
   H T Q N Q P L T H F V L Y S S A A
   GOCACCTCGGCAGCCCCCAAGCCAACTACGCGCGCCAACCACCAACAACACCCCTT 4600
25
   ATLGSPGQAUYAAAUAF
   COTOGREGOCOTOGOCACCCACÔGOCACACCCALGGA CARCOCCECACOA 4600
   LDALATHRETÇGQPAT
   CCATCGCCTGGGGGATGTGGCACACCACCACCACACTCACCAGCCAACTC 4700
   TIAWGMWHTTTTLTSQL
   ACCGACAGCGACCGCGACCGCATCCGCCGCGGCGGCTTCCTGCCGATCTC 4750
   TOSORDRIRRGGFLFIS
   GGACGACGAGGGCATGC
    D D E G M
35
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The *NheI-XhoI* hybrid FK-506 PKS module 8 containing the AT domain of module 12 of rapamycin is shown below.

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GOATGOGGCTGTACGAGGOGGCACGGCGCACGGAAGTCCCGTGGTGGTG-50
     M R L Y E A A R R T G S P V V V
   40
   A A A L D D A P D V P L L R G L R
   GOSTACGACOGTOCGGCGTGCCGCCGTCCGGGAACGCTCTCTCTCGCCGACC 150
    RIT T V R R A A V R E R S L A D
   GOTEGOEGTGETGECCGACGACGACGACGCCCCACGCCTCCCTTCGCCTTCG 200
   R S P C O P T T S A P T P P S F S
   TCCTGGAACAGCACCGCCACCGTGCTCGGCCACCTG3GCGCGAAGACAT 250
   S W N S T A T V L G H L G A E D I
   PATTFKELGIES LTA
   TOCASCTGCGCANOGCGCTGRCCACGGCGACCGGCGTACGCCTCRACGCC 350
50
   V Q L R N A L T T A T G V R L N A
   ACAGOGGTOTTOGACTTTCCGACGCGCGCGCGCGCTCSCCGCGAGACTCGG 400
   TAVFDFPTPRALAARLG
   CGACGAGCTGGCCGGTACCCGCGCGCGCCCCTCGCGGCCCGGACCGCGGCCA 450
   DELAGTRAPVAARTAA
55
   T A A A H D E P L A I V 3 M A C R
   CTGCCGGGGGGGGTGGGGTCGCCACAGGAGCTGTGGCGTCTCGTCGCGTC 550
    LPGGVASPQELNFLVAS
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	03p1A004A030AT0A056A3TT005033A003055555555555555	600
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5		
	 B F F F D D B A D G F D A A F F B BATTABUTCHUMASSUCCHUSCHUSCHUSCHUSCHUSCHUSCHUSCHUSCHUS	750
10	TO BUT IN THE REPORT OF A CONTROL OF THE RESERVENCE OF THE RESERVE	i i i
• •	- W - Z - Z - W - R - A - F - R - B - A - R - Z - Z - F - Z - A - G - Z - Z - F - Z - A - G - Z - Z - Z - Z - Z - Z - Z - Z - Z	2 2 2
	PROPRIES TO COMMON ACCES OF THE GOOD SACAS GOTTOS CASATTAL OF THE GOOD SACAS GOTTOS CASATTAL SERVICES OF THE GOOD SACATTAL SACATTAL SERVICES OF THE GOOD SACATTAL SACATTA	306
15	GROTGOTOCOGGOCOTOCOTACTACTACGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGOCOTOTOGGAGGAGGOCOTOTOGGAGGAGGAGGOCOTOTOGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGAGGA	950
	STCACGETCGACACGCCTGCTCGTCGTCGTCGCCCCGCACCAGGC	1000
20	AGGGCAGTCCCTGCGCTCGGGCGAATGCTCGCTCGCCCTGGTCGGCGCTG	1050
	TOACGGTGATGGCGTCGCCGGGGGGATTCGTCGAGTTCTCCCGGGCAGCGC	1300
25	GGGCTOGCGCGGACGGCCGGGCGGGCGGGCGGGCGGGCGGGCGGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGCGACGA	1150
25	TACGAGOTTCGCCGAGGGCGCCGGTGCCCTGGTGGTCGAGGGGCTCTCCG T S F A E G A G A L V V E R L S	1200
	ACGCGGAGCGCCACACCGTCCTCGCCCTCGTACGCGGCTCCGGG PAERHGHTVLALVRGSAA	1150
3()	GCTAACTCCGACGGGGGGTCGAACGGTCTGTCGGGGGGGAACGGCCCCTC A N S D G A S N G L S A F N G P S	1300
	CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCCG 2 E R V I H Q A L A N A K L T F	1350
	COGATGTOGACGOGGTOGAGGCGCACGGCACCGGCCTCGGCGAC A D V D A V E A E G T 3 T E L G C	1400
35	CCCATCGAGGCGCAGGCGCTGCTCGCGACGTACGGACAGGACCGGGCGAC P I E A Q A L L A T Y G Q D R A T	1450
	GCCCCTGCTGCTCGCTCGCTGAAGTCGAACATCGGGCACGCCCAGGCCG F L L L G S L K S N I G H A Q A	1500
40	CSTCAGGGGTCGCGGGATCATCAAGATGGTGCAGGCATCCGGCACGGG	1850
	SAACTGCCGCCGACACTGCACGCGGACGAGCCGTCGCCGCCGCACGTCGACTG E L P P T L H A D E P S P H V D W	1600
	GACGGCCGGTGGAGCTCCTGACGTCGGCCGGCCGGGGGAAAAAAAA	1650
45	COGGTCGCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCG	1700
	AACGCCCACATCATCCTTGAGGCAGGACCGGTCAAAACGGGACCGGTCGA N A H I I L E A S P V E T S P V E	1750
50		1800
		1850
		1900
55		1950
	AGACACTGGGCGGGGTACGCACTTCACCCACCGGGGCGTACTGCTCGGG	2000
60	GACACCGTCATCGGCGCTCCCCCCGCGGGACCAGGCGAACTCGTCTT. D T V	2050

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        proproduce tropic de la company de la compan
        an armopakki adkaan darmometnogaan actaakki cefeaa ana 1250
          PFAMLTALFOLDESWS
        TA CGN 2003A 6 9 2 G STGA T G 9 G DANTT 26 GTG G G T GA G CTTG 9 G G ACT 9 CG - 2 G 3 9
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         NATISTI STOCCO SIGSTENEST CONTISCA COAT SCONSOA COTTIGGT STOCG S
         Y V S 3 T W S L B D A ? ? L J .
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          RABIM DALPASSUM VA
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        Podosononorska skoskoska podogagodaracha sanakadar anaska 19450
          PYSETEABAVISESV
       GBCCGTGCTGCAGGCCGCGGAGGGGCTGGGGAAGTGGACGCGGCTGGTGA 2550
20
         AVL 2A A E G L G K W T R L A
        DCAGOCACGCCTTCCATTCCGCCCCTMTGGAACCCATGCTGGAGGAGCCC
         S H A F H S A R M E P M L E E F
       CGGGGGGTCGCGAAGSCCTGACCTACCGGACGCCGCAGGTCTCCATGGC 2650
        RAVASSLTYRTPQVSKA
25
       CGTTGGTGATCAGGTGACCACCGCTGAGTACTGGGTGCGGCAGGTCCGGG 2700
         V G D D V T T A B Y W V B Q V B
       ACACGGTCTSSTTCGGCGAGCAGGTGGCCTCGTACGAGGACGCCGTGTTC 2750
        I T V P F G E Q V A S Y E D A V F
       STOGASOTGGGTGCGACCGGTCACTGGCCCGCCTGGTCGACGGTGTCGC 2800
30
       V E L G A D R S L A R L V D G V A
       GATGCTGCACGGCGCCACGAAATCCAGGCCGCGATCGGCCCCTGGCCC 2850
          M L H 3 D H E I Q A A I G A L A
       ACCTGTATGTCAACGGCTCACGGTCGACTGGCCCGCGCTCCTGGGCGAT 2900
       H L Y V N G V T V D W P A L L G D
35
       GOTOOGGCAACAOGGGTGOTGGAOCTTOCGACATAOGOCTTCCAGCAOCA 2950
        A PIATR V L D L P T Y A F Q H Q
       GCGCTACTSSCTCGAGTCGGCTCCCCCGGCCACGGCCGACTCGGGCCACC 3000
          RYWLESAPPATADSGH
       COGTCCTCGGCACCGGAGTCGCCGTCGCCGGGTCGCCGGGGCGGGGTGTTC 3050
40
       PVLGTGVAVAGSPGRVF
       ACGGGTCCCGTGCCGCGGGTGCGGACCGCGCGGTGTTCATCGCCGAACT 3100
       TGPVPAGADRAVFIAEL
       GGCGCTCGCCGCCGCCGACGCCACCGACTGCGCCACGGTCGAACAGCTCG 3150
         A L A A A D A T D C A T V E Q L
45
       D Y T S Y P G G S A R G R A T A Q
       ACCTSGGTCCATGAACCCCCCCCCCACGGGGGGGGCGCCCCTTCACCGTCCA 3250
        T W Y D E P A A D G F R R F T V H
       CACCCCCTTGGCGACGCCCCTGGACGCTGCACGCCGAGGGGGGTTCTCC 3300
50
        TRVGDAPWTLHAEGVA
       GDCCCGGCCGCGTGCCCCAAGCCGAAGCCGTCGACACGCCTGGCCCCCG 3359
       RPGBWFQPEAVDTAWPF
       DDGGGGGGGTGCCCGGGGACGGGGCTGCCCGGGGGGGTGGCGACGCGGGA 3400
        P G A V P A D G L P G A W R R A D
       CORGSTOTTOSTOGRAGOOGRAGTOGACAGOOOTGACGGOTTOGTGGCAC 3450
55
          Q V P V B A B V D S P D G F V A
       ACCCCGACCTSCTCGACGCGCTCTTCTCCGCGGTCGGCGACGGGAGCCGC 3500
       H B D L L B A V F S A V G D G S R
       CAGCCGACCGGATGGCGCGACCTCGCGGTGCACGCGTCGGACGCCACCGT 3550
60
        Q P T G W R D L A V H A S D A T V
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antradeo a porte entra de nacida CARGNA STA STA STA CETTRA SOCIOCADOS (2.66). LIVANIA DE LITERA RODER DE SERVER EL A :Troskogg:goddskkragodGTQCTQAQQGTQAQQqqqqqqqqqqqq fanéastruberreeduadgebaarreedaanaarreedaaderreetri artij 327.Gng.gggmng695g.ggg68AGG66ChCTh0GhCG6CGCGhCah2ah26 3457 LEWLFTABABTIGACE TACTOSA GRACIA CACOTTO A COACCACA CACACO COA ACCACA COCCAA (C. 1980). 1 | F | E | 2 | T | T | 1 | T | A | T | 8 | F | C | E | E | C 10 SEGE : ACCEPTACO EN EN CARCA COCA CARCA COLA CARCA CAR A BEST CONTRACTOR A CARCARCO ACCOMENTACIÓN DA CARCARDO ACCARCARDO ACARCARDO ACCARCARDO A TORRUGATOCA DA CORCOR CONCORDA COCOCA CONTRA CORCORDA CONTRA CONTRA CONTRA CONTRA CONTRA CONTRA CONTRA CONTRA CO 15 ACCORCAL LICACAMANCAMACHOCOCGGCCSCATICTACCTCATICGALA 1/4/100 TRTAQNEHPGRIHLIE BOADOADODOONOROCCACTOCCCCTCACCURACTOROCACCACC 4050 20 HHPHTPLPLTQLTTLE CONCARCODORACOROGOCATOCTCATOROGGGGGGTTCCGGGACCOTCG 4200 25 INPNHAILITSGSGTI COGGCATOTTOGGCCGCCACCTCAACCACCCCCACACCTACCTCCTCTCC 4250 A G I L A R H L N H P H T Y L L S GGCACACCACCCCCACCACCCCGGCACCCACATTCCCCTGCGACCT 4390 R T P P P T T P G T H I P C D L 30 CACCGACCCCAACCCAAATCACCCAAGCCCTCACCCACATACCACAACCCC 4350 T D P T Q I T Q A L T H I F Q P TOACOGGOATOTTOOACACCGCCGCCACCCTCGACGACGCCACCCTCACC 4400 D T G D F H T A A T L D D A T D 35 N L T F Q H L T T T L Q F H A D A GSCCTSGCAGGTCGAGGAGGAGGGAAAAGGAAGCGTCAGGGAGTTCS 4500 A W H L H H H T Q N Q P L T H F TOOTSTASTOOAGOGGGGGGGGGGGGTCGGGAGGGGGGGGAAGGGAAC 4850 40 LYSSAAATLGSPGQAU TACGCCGCCCAACGCCTTCCTCGACGCCCTCGCCACCCGCCACCGCCACAC 4600 Y A A A N A F L D A L A T H R H CCAMBGACAACCCGCCACCATCGCCTGGGGCATGTGGCACACCACCA 4650 Q G Q P A T T I A W G M X H T T CCACACTCACCAGCCAACTCACCGACAGCGACCGCGACCGCATCCGCCGC 4700 45 TTLTSQLTDSDFCRIRE GGCGGCTTCCTGCCGATCTCGGACGACGAGGGCATGC GGFLFISDDEGM

The *Nhel-Xhol* hybrid FK-506 PKS module 8 containing the AT domain of module 13 of rapamycin is shown below.

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TATAMANA BOAD BOOMO DOTACTO BEOCHADOTO SOCIODA A GAMENT (25)
A TAMAN OLO TAMANT (VILLO) BALLO A BEOCHA
                                  GADBA GUADBTTOAABGAADTGGBOATGBACTOBCTCACCBCBB 300
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                FOR FOR A CONTROL OF THE PROPERTY OF THE PROPE
                     3970770326<mark>0777</mark>0362333336303030307030793826932693
                   TAVESFETERALAAPLS
                     $$$$$A$$$$$$$$$#\$$$$$$##\$$$$$$$$$$$$$$#\$$$$$$#\$$$$#\$$$$#\$$$$#\$$$$#\$$$
 10
                      T D L A G T A A F T A A F T A A
                 A A A B D E P S A I T B M A
                L P 3 G V A S P Q E 1 W R L V A C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G R C G 
 15
                     ANG BOTOTARGMORDEGACE BOGACGOOMTOGGGAAGACGTT0GT0055 (5)
                Q A L Y S P D P D A 1 G K T F Y
                .
CA CÉ POUS ETT DOTTE GA OGSTGOGA COGOTTO GA CGE GA COTT ETT DE S
 20
                B 3 3 F L D G A T G F D A A F F 3
                GATOR DOCOGRAGOCA GOODA TAGOR DOCOGOR SON ROS DA SON COMO POR CONTRO DA COMO POR CONTRO DA COMO POR CONTRO DA COMO POR C
               I F F K E A L A M D F Q Q F V 1
Tgsasacstootgggaggggttegaaageggggatcacscossgacsgcs 800
                L E T S W E A F E S A G S T F S A
25
               GCGCGGGGCACACCGGCGTGTTCATCGGCGCGTTCTCCTACGGGTA 850
                ARGSDTGVFIGAFSYS
                COSCA DESETECEGATACCAACEGOTTOGECGOGACASEGTES DAGACCA - 900
                   G T G A D T N G F G A T G S Q
               GCGTGCTCTCCGGGCCGCCTCGTACTTCTACGGTCTGGAGGGCCCTTCG 950
30
               S T L S G R L S Y F Y G L E G F S
               STCACGSTCGACACCGCCTCGTCGTCGTCACTGGTCGCCCTGCACCAGGC 1000
                Y T V D T A C S S S L V A L H Q A
               AGGGCASTOCOTGCGCTCGGGGGGGAATGCTCGCTCGCCCTGGTCGGCGGCGGTG 1050
                  35
               TONOSSTANOSOSTOSCOCGGGGGMTTCSTCCNATTCTCCCGGGGAGCGC 1100
              V T U M A S P G G F V E F S N Q R
               33887738939<del>96</del>34666699666884466937775883606666666668888
               J L A P E G R A K A F G A G A C G
               40
                 T S F A E G A G A L V V E R I
              ACGOGGAGGOCOACACGTCCTCGCCCTCGTACGCGGCTCCCCCG 1250
              DAERHGHTVLALVRGSA
               SCTAR STOOGA CGGCGGTCGAACGGTCTGTCGGCGCCGAACGGCCCCTC 1300
                A N S D G A S N G L S A P N G P S
45
               CCAGGAACGCGTCATCCACCAGGCCCTCGCGAACGCGAAACTCACCCCCG 1350
                 QERVIRQALAHAKLT F
              A C V D A V E A H G T G T R L G D
               DECATOGACESCASGOSCTGCTSSCGACGTACGGACAGGACCGGGGGAC 1450
50
                 FIEAÇALLATYGÇDAAT
                           OTGCTGCTGGGCTGGCTGAAGTCGAAGATCGGGGCACGCCCAGGCCG 1500
                    F t t t t g s f k s H i g H a Q a
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55
                ELPPTLHADEPSPHVDW
               3A MGGCCGGTGCGCTGCAGCTGCTGACGTGGGCGGGCGTGGCCGGGGGA 1650
                  CCGGTCGCCCGCGCGCGCGCGTGCCGTCGTCGTTCGGCGTGAGCGGCACG 1700
60
               T G R F R R A A V S S F G V S G T
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	WARRITHA CATESTT BAGG CAGGACGGST CAAAA CGGGACGGST GA	1780
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5	SACCECTOCOCOCOSOSOSOSOSOSOSOSOSOSOSOSOSOSOSOS	1350
	TOSTGTEGGGGGGTTECCTGGAGGAGGAGGAGATCGGGGGGGCCT	
10	General Tarter as Acadego de as General Sacras Sacras de Constantes de C	
10	A SACACTOS DO COSO STA COCA CTTCA COCACO SAGO COTACTOS TO COCACO SAGO COTACTOS CO	2000
	O T L A R R T H F T H F A M L L G GAUACOGTOATOGGGGGTOCGGGGGGGGGGGGGGGGGGGGGGG	2050
15	E T W I G A P E A D Q A D E L W E montotacteographic montotacteographic montotacteographic description of the contotacteographic montotacteographic description of the contotacteographic descriptin description of the contotacteographic description of the contot	2130
	V Y S C C G C P H P A M G E V D CORATTOSTOSTATTSTTOSCOSASOSGATGGCGGCGGCGGGG	2150
20	A L S S V V F A E R M A E C A A A TTGCGCGGAGTTCGGGAGTGGGATCTGTTCACGGTTCTGGATGATCCGGC	
20	L R E F V D W D L F T V L D D P A SSTSGTGGACGGGTTGATGTGGTCCAGCCCGCTTCCTGGGCGATGATGG	
	V V D R V D V V Q P A S W A M M TTTCCTTGGCGGGTGTGGCAGGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGCGGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTGTG	2300
25	V S L A A V W Q A A G V R P D A V ATCGCCATTCGCAGGTGAGATCGCCGCAGCTTGTGTGGCGGGTGCGT	2350
	I G H S Q G E I A A A C V A G A V GTCACTACGCGATGCCGCCCGGATCGTGACCTTGCGCAGCCAGGCGATCG	2400
30	ELRDAARIVTLRSQAI cccssgcctrgcggggggggggggatggcatcgtcgcctgcccscg	2450
50	A R G L A G R G A M A S V A L P A CAGGATGTCGAGCTGGCGGGCCCACAACGGGCC O O V E L V D G A W I A A H N G P	2500
	Q D V E L V D G A W I A A H N G P CGCCTCCACCGTGATCGCGGGGGCCCCCGGGAGCGGTCGACCATGTCCTCA A S T V I A G T P E A V D H V L	2550
35		2600
	GOOTOGOACACCCCGCGACCTGATCCGCGACGACTACTCGACAT A S H T P H V E L I R D E L L D I	2650
40	CACTAGOGACAGCTCGCAGACCCCGCTCGTGCCGTGCCTGTCGACCC T S D S S S O T P L V P W L S T	2700
.0	TEGROGECACCTEGGTCGACAGCCCGCTGGACGGGGAGTACTGGTACCGG	2750
	AACCTGCGTGAACCGGTCGGTTTCCACCCCGCCGTCAGCCAGTTGCAGGC N L R E P V G F H P A V S Q L Q A	2800
45	CCAGGGGGACACCGTGTTCGTCGAGGTCAGCGCCAGCCCGGTGTTGTTGC	2850
	AGGCGATGGACGATGTCGTCACGGTTGCCACGCTGCGTCGTGACGAC	2900
50	GGCGACGCCACCCGCATGCTCACCGCCCTGGCACAGGCCTATGTCCACGG GCGCACGCCACCGCCCTGGCACAGGCCTATGTCCACGG GCGCACGCCACCACGCCTGCCACAGGCCTATGTCCACGG	2950
	COTCACOGTOGACTGGCCCCCACCACCACCACCACCACCACCACCACCACCACC	3000
	TGGACCTTCCGACCTACGCCTTCCAACACCAGCGGTACTGGCTCGAGTCG	3050
55	SOTTOCCCGGCCACGGCGACTCGGGCACCGGAGT A P F A T A D S G H P V L G T G V	3100
	TRECONTOR OF SET OF CONTROL OF THE C	3150
	GTGCGGACCGCGCGGTGTTCATCGCCGAACTGGCGCTCGCCGCCGCCGAC	3200
60	G A D R A V F I A E L A L A A D	

		3250
	DOBATORSCOCGOGOSA GOGOCÁCO GOGO A COTOGO TOCATORA A TOCOG	3350
	THE ABORATA TOWNS E	
5	- COGDOGA GGGGGGGGGGGGCCCCCCCCCCCCACA DOGGGGGGGGGGA TGDG	3350
	A A D G R R R T V B T A V G D A	2770
	ACCCCCTCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC	3400
		3900
	PWTLHAEGVLRPGRVPQ	
1.0	3000GAAGCCGTCAACACCGCCTGGCCCCCCCGCGGGGGGGGGG	3450
10	PEAVDTAWPPPGAVPA	
	ACGGGCTGCCCGGGGGCGTGGCGACGCGGGGACCAGGTCTTCGTCGAAAGCC	3500
	B G L P G A W R R A D Q V F V E A	
	GAAGTOGACAGOGOTGACGGOTTCGTGGCACAGCGGACCTGGTGGACGG	3550
	EVDS 2 DG FVA H P D L L D A	
15	93707707000000700000A0000A0000A0000A70000A700000A	3600
		2552
	ADDITOGOGGTGCACGCGTCGGACGCCACCGTGCTGCGCGCGCCTGCCT	3650
	D L A V H A S D A T V L R A C L T	
	OBCCGCGACACTGCGTGGAGGTGGGAGCTCGACGGTGCGGAAT	3700
20	BRDSGV ELAA FDGAGM	
	GCCGGTGCTCACCGCGGAGTGGGTGACGCTGGGCGAGGTCGCGTCGGCAG	3750
	PULTAESVTLGEVASA	
	GOGGATOCGACGACTCGGACGCTCTGCTTCGGCTTGAGTGGTTGCCGGTG	3800
	F G S D E S D G L L R L E W L F V	2000
25		2020
~)	GCGGAGGCCCACTACGACGGTGCCGACGAGCTGCCCGAGGGCTACACCCT	3850
	A E A H Y D G A D E L P E G Y T L	
	CATCACCGCCACACACCCCGACGACCCCGACGACCCCACCAACCCCCACA	3900
	ITATHPODPDDPTNPH	
	ACACACCCACACGCACCCACACACACACACGCGCGTCCTCACCGCCCCC	3950
30	RTPTRTHTQTTRVLTAL	
	CAACACCACCTCATCACCACCAACCACCCTCATCGTCCACACCACCAC	4000
	Q H H L I T T N H T L I V H T T T	
	CGACCCCCCAGGCCCCCGCTCACCGCCTCACCCGCACCACACAAAACG	4050
		4000
35		1100
20	AACAGGGGGGGGATCCACCTCATCGAAACCCACCACCCCCACAGGGGA	4100
	EHPGRIHLIETHHPHTP	
	CTCCCCTCACCCAACTCACCACCCTCCACCAACCCACCTACGCCTCACC	4150
•		
	ACACCACCOTCCACACACCCCCCCCCCCCCCCCCCCCCC	4200
40	NNTLHTPHLTPITTHH	
	ACACCACCACAACCACCCCAACACCCCACCCCTCAACCCCAACCACC	4250
	N T T T T P N T P P L N P N H A	
	ATCCTCATCACCGGCGGCTCCGGCGCCCCCCCCCA	430n
		4300
15	I I I T G G S G T L A G I L A R H	4050
45	CCTCAACCACCCCACACCTACCTCCTCTCCCGCACACCACCACCCCCCA	4350
	LNHPHTYLLSRTPPP	
	CCACACCCGGCACCCACATCCCCTGCGACCTCACCGACCCCACCCA	4400
	TTPGTHIPCDLTDPTQI	
	ACCCAAGCCCTCACCCACATACCACAACCCCTCACCGGCATCTTCCACAC	4450
50	TQALTHIPQPLTGIFHT	
		4500
	A A T L D D A T L T N L T P Q H	1555
		4550
	TCACCACCACCCTCCAACCCAAAGCCGACGCCGCCTGGCACCTCCACCAC	4550
	L T T T L Q P K A D A A W H L H H	
55	-1,41,003,622,000,2,000,000,000	4600
	HTQNQPLTHEVLYSSAA	
		4650
	ATLGSP6QANTAAANA	
	TOOTOGACGCCTCGCCACCCACCGCCACACCCAAGGACAACCCGCCACC	4700
60	F L D A L A T H R H T Q G Q P A T	
00		

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Example 3

Recombinant PKS Genes for 13-desmethoxy FK-506 and FK-520

The present invention provides a variety of recombinant PKS genes in addition to those described in Examples 1 and 2 for producing 13-desmethoxy FK-506 and FK-520 compounds. This Example provides the construction protocols for recombinant FK-520 and FK-506 (from *Streptomyces* sp. MA6858 (ATCC 55098), described in U.S. Patent Nos. 5,116,756, incorporated herein by reference) PKS genes in which the module 8 AT coding sequences have been replaced by either the *rapAT3* (the AT domain from module 3 of the rapamycin PKS), *rapAT12*, *eryAT1* (the AT domain from module 1 of the erythromycin (DEBS) PKS), or *eryAT2* coding sequences. Each of these constructs provides a PKS that produces the 13-desmethoxy-13-methyl derivative, except for the rapAT12 replacement, which provides the 13-desmethoxy derivative, i.e., it has a hydrogen where the other derivatives have methyl.

Figure 7 shows the process used to generate the AT replacement constructs. First, a fragment of ~4.5 kb containing module 8 coding sequences from the FK-520 cluster of ATCC 14891 was cloned using the convenient restriction sites Sacl and SphI (Step A in Figure 7). The choice of restriction sites used to clone a 4.0 - 4.5 kb fragment comprising module 8 coding sequences from other FK-520 or FK-506 clusters can be different depending on the DNA sequence, but the overall scheme is identical. The unique SacI and SphI restriction sites at the ends of the FK-520 modifie 8 fragment were then changed to unique Bgl II and NsiI sites by ligation to synthetic linkers (described in the preceding Examples, see Step B of Figure 7). Fragments containing sequences 5' and 3' of the AT8 sequences were then amplified using primers, described above, that introduced either an AvrII site or an NheI site at two different KS/AT boundaries and an XhoI site at the AT/DH boundary (Step C of Figure 7). Heterologous AT domains from the rapamycin and erythromycin gene clusters were amplified using primers, as described above, that introduced the same sites as just described (Step D of Figure 7). The fragments were ligated to give hybrid modules with in-frame fusions at the KS/AT and AT/DH boundaries (Step E of Figure 7). Finally, these hybrid modules were ligated into the BamHI and PstI sites of the

KC515 vector. The resulting recombinant phage were used to transform the FK-506 and FK-520 producer strains to yield the desired recombinant cells, as described in the preceding Examples.

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The following table shows the location and sequences surrounding the engineered site of each of the heterologous AT domains employed. The FK-506 hybrid construct was used as a control for the FK-520 recombinant cells produced, and a similar FK-520 hybrid construct was used as a control for the FK-506 recombinant cells.

Hecologous AT	Enzyme	Location of Engineered Site
FK-506 AT8	AvrII	GGCCGTdbddbdCGTCGCGGCGTCTCGTCGTTC
(hydroxymalonyl)		G R P R R A A V S S F
(11) 42 011) 12141 011) 1)	NheI	ACCCASCATCCCGGGGATGGGTGAGCGggttggcC
	11/1/01	TQHFAMGERLA
	V1 Y	TACGCCTTCCAGCGGCGGCCTACTGGatcgag
	Xhol	YAFQARPYWIE
rapamycin AT3	AvrII	GACCGG_cccctCGGGCGGGCGTGTCGTCCTTC
(methylmalonyl)		D R P R R A G V S S F
	Nhel	TGGCAGTGGCTGGGGATGGGCAGTGCoctacaG
		W Q W L G M G S A L R
	XhoI	TACGCCTTCCAACACCAGCGGTACTGGgtcgag
rapamycin AT12	AvrII	GGCCGA <u>acacac</u> CGGGCAGGCGTGTCGTCCTTC
(malonyl)		TCGCAGCGTGCTGGCATGGGTGAGGAactggcC
	NheI	S Q R A G M G E E L A
		TACGCCTTCCAGCACCAGCGCTACTGGctcgag
	<i>Yho</i> I	Y A F Q H C R Y W L E
DEBS AT1	AvrII	GCGCGAecgcgcGGGGGGGGGTCTCGTCGTTC
		ARPRAGVSSE
(methylmalonyl))	TGGCAGTGGGGGGGCATGGCCGTCGAggtggtC
	NheI	W Q W A G M A V D L L
		TACCCGTTCCAGCGCGAGCGCGTCTGGctcgaa
	XhoI	Y P F Q R E R V W L E
DEBS AT2	AvrII	GACGGG <u>atacqc</u> CGGGCAGGTGTGTCGGCGTTC
(methylmalonyl)		DGVRRAGVSAF
(2)	NheI	GCCCAGTGGGAAGGCATGGCGGGGAgttgttG
	1,,,,,,	AQWEGMARELL
	\ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \	TATCCTTTCCAGGGCAAGCGGTTCTGGctgctg
	XhoI	Y P F Q G K R F W L L

The sequences shown below provide the location of the KS/AT boundaries chosen in the FK-520 module 8 coding sequences. Regions where AvrII and NheI sites were engineered are indicated by lower case and underlining.

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The sequences shown below provide the location of the AT/DH boundary chosen in the FK-520 module 8 coding sequences. The region where an *MoI* site was engineered is indicated by lower case and underlining.

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The sequences shown below provide the location of the KS/AT boundaries chosen in the FK-506 module 8 coding sequences. Regions where AvrII and NheI sites were engineered are indicated by lower case and underlining.

TOGGOCAGGOOGTGGCCGGGGGGGCCGT<u>eagag</u>aCGTGCGGGGGTCTCGTCGTTCGGG 30 S A R P W P R T G R P R R A A V S S F G GTGAGCGGCACCAACGCCCACATCATCCTGGAGGCCGGACCCGACCAGGAGGAGCCGTCG V S G T N A H I I L E A G P D Q E E P S GCAGAACCGGCCGGTGACCTCCCGCTGCTCGTGTCGGCACGGTCCCCGGAGGCACTGGAC A E F A G D L P L L V S A R S P E A L D GAGCAGATCGGGGGGCTGGGGGACTATCTCGACGCCGCCCCGGCGTGGACCTGGCGGCC 35 EQIGRLRDYLSAAPSVCLAA GTGGGGGGACACTGGCCACGGGTACGCACTTCTCCCACCGCGCGTACTGCTCGGTGAC V A R T L A T R T H F S H R A V L L G D MCCGTCATCACCGCTCCCCCCGTGGAACAGCCGGGGGGGGTCGTCTTCGTCTACTCGGGA TVITAPPVEQPGELVFVYSG CAGGGCACCCAGCATCCCGCGATGGGTGAGCGgctcacCGCAGCCTTCCCCGTGTTCGCC Q G T Q H P A M G E R L A A A F P V F A GACCOGGACGTACCCCCTACGCCTTCCAGCGGCGCCCTACTGGATCGAGTCCGCGCCG D P D V P A Y A F Q R R P Y W I E S A P 45

The sequences shown below provide the location of the AT/DH boundary chosen in the FK-506 module 8 coding sequences. The region where an *XhoI* site was engineered is indicated by lower case and underlining.

GACCOGGACGTACCOGCCTACGCCTTCCAGCGGCGCCTACTGGategagTCCGCGCGC

Example 4

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Replacement of Methoxyl with Hydrogen or Methyl at C-15 of FK-506 and FK-520

The methods and reagents of the present invention also provide novel FK-506 and FK-520 derivatives in which the methoxy group at C-15 is replaced by a hydrogen or methyl. These derivatives are produced in recombinant host cells of the invention that express recombinant PKS enzymes the produce the derivatives. These recombinant PKS enzymes are prepared in accordance with the methodology of Examples 1 and 2, with the exception that AT domain of module 7, instead of module 8, is replaced. Moreover, the present invention provides recombinant PKS enzymes in which the AT domains of both modules 7 and 8 have been changed. The table below summarizes the various compounds provided by the present invention.

	Compound	C-13	C-15	Derivative Provided
	FK-506	hydrogen	hydrogen	13, 15-didesmethoxy-FK-506
	FK-506	hydrogen	methoxy	13-desmethoxy-FK-506
15	FK-506	hydrogen	methyl	13,15-didesmethoxy-15-methyl-FK-506
	FK-506	methoxy	hydrogen	15-desmethoxy-FK-506
	FK-506	methoxy	methoxy	Original Compound FK-506
	FK-506	methoxy	methyl	15-desmethoxy-15-methyl-FK-506
	FK-506	methyl	hydrogen	13,15-didesmethoxy-13-methyl-FK-506
20	FK-506	methyl	methoxy	13-desmethoxy-13-methyl-FK-506
	FK-506	methyl	methyl	13,15-didesmethoxy-13,15-dimethyl-FK-506
	FK-520	hydrogen	hydrogen	13, 15-didesmethoxy FK-520
	FK-520	hydrogen	methoxy	13-desmethoxy FK-520
	FK-520	hydrogen	methyl	13,15-didesmethoxy-15-methyl-FK-520
25	FK-520	methoxy	hydrogen	15-desmethoxy-FK-520
	FK-520	methoxy	methoxy	Original Compound FK-520
	FK-520	methoxy	methyl	15-desmethoxy-15-methyl-FK-520
	FK-520	methyl	hydrogen	13,15-didesmethoxy-13-methyl-FK-520
	FK-520	methyl	methoxy	13-desmethoxy-13-methyl-FK-520
30	FK-520	methyl	methyl	13,15-didesmethoxy-13,15-dimethyl-FK-520

Example 5

Replacement of Methoxyl with Ethyl at C-13 and/or C-15 of FK-506 and FK-520

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The present invention also provides novel FK-506 and FK-520 derivative compounds in which the methoxy groups at either or both the C-13 and C-15 positions are instead ethyl groups. These compounds are produced by novel PKS enzymes of the invention in which the AT domains of modules 8 and or 7 are converted to ethylmalonyl specific AT domains by modification of the PKS gene that encodes the module. Ethylmalonyl specific AT domain coding sequences can be obtained from, for example, the FK-520 PKS genes, the niddamycin PKS genes, and the tylosin PKS genes. The novel PKS genes of the invention include not only those in which either or both of the AT domains of modules 7 and 8 have been converted to ethylmalonyl specific AT domain but also those in which one of the modules is converted to an ethylmalonyl specific AT domain and the other is converted to a malonyl specific or a methylmalonyl specific AT domain.

Example 6

Neurotrophic Compounds

The compounds described in Examples 1 - 4, inclusive have immunosuppressant activity and can be employed as immunosuppressants in a manner and in formulations similar to those employed for FK-506. The compounds of the invention are generally effective for the prevention of organ rejection in patients receiving organ transplants and in particular can be used for immunosuppression following orthotopic liver transplantation.

These compounds also have pharmacokinetic properties and metabolism that are more advantageous for certain applications relative to those of FK-506 or FK-520. These compounds are also neurotrophic; however, for use as neurotrophins, it is desirable to modify the compounds to diminish or abolish their immunosuppressant activity. This can be readily accomplished by hydroxylating the compounds at the C-18 position using
established chemical methodology or novel FK-520 PKS genes provided by the present invention.

Thus, in one aspect, the present invention provides a method for stimulating nerve growth that comprises administering a therapeutically effective dose of 18-hydroxy-FK-520. In another embodiment, the compound administered is a C-18,20-dihydroxy-FK-520 derivative. In another embodiment, the compound administered is a C-13-desmethoxy and/or C-15-desmethoxy 18-hydroxy-FK-520 derivative. In another embodiment, the compound administered is a C-13-desmethoxy and/or C-15-desmethoxy 18,20-dihydroxy-FK-520 derivative. In other embodiments, the compounds are the corresponding analogs of

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FK-506. The 18-hydroxy compounds of the invention can be prepared chemically, as described in U.S. Patent No. 5,189,042, incorporated herein by reference, or by fermentation of a recombinant host cell provided by the present invention that expresses a recombinant PKS in which the module 5 DH domain has been deleted or rendered non-functional.

The chemical methodology is as follows. A compound of the invention (~200 mg) is dissolved in 3 mL of dry methylene chloride and added to 45 µL of 2,6-lutidine, and the mixture stirred at room temperature. After 10 minutes, tert-butyldimethylsilyl trifluoromethanesulfonate (64 µL) is added by syringe. After 15 minutes, the reaction mixture is diluted with ethyl acetate, washed with saturated bicarbonate, washed with brine. and the organic phase dried over magnesium sulfate. Removal of solvent in vacuo and flash chromatography on silica gel (ethyl acetate: hexane (1:2) plus 1% methanol) gives the protected compound, which is dissolved in 95% ethanol (2.2 mL) and to which is added 53 μL of pyridine, followed by selenium dioxide (58 mg). The flask is fitted with a water condenser and heated to 70°C on a mantle. After 20 hours, the mixture is cooled to room temperature, filtered through diatomaceous earth, and the filtrate poured into a saturated sodium bicarbonate solution. This is extracted with ethyl acetate, and the organic phase is washed with brine and dried over magnesium sulfate. The solution is concentrated and purified by flash chromatography on silica gel (ethyl acetate: hexane (1:2) plus 1% methanol) to give the protected 18-hydroxy compound. This compound is dissolved in acetonitrile and treated with aqueous HF to remove the protecting groups. After dilution with ethyl acetate, the mixture is washed with saturated bicarbonate and brine, dried over magnesium sulfate, filtered, and evaporated to yield the 18-hydroxy compound. Thus, the present invention provides the C-18-hydroxyl derivatives of the compounds described in Examples 1 - 4.

Those of skill in the art will recognize that other suitable chemical procedures can be used to prepare the novel 18-hydroxy compounds of the invention. See, e.g., Kawai et al., Jan. 1993, Structure-activity profiles of macrolactam immunosuppressant FK-506 analogues, FEBS Letters 316(2): 107-113, incorporated herein by reference. These methods can be used to prepare both the C18-[S]-OH and C18-[R]-OH enantiomers, with the R enantiomer showing a somewhat lower IC₅₀, which may be preferred in some applications. See Kawai et al., supra. Another preferred protocol is described in Umbreit and Sharpless, 1977, JACS 99(16): 1526-28, although it may be preferable to use 30 equivalents each of

 SeO_2 and t-BuOOH rather than the 0.02 and 3-4 equivalents, respectively, described in that reference.

All scientific and patent publications referenced herein are hereby incorporated by reference. The invention having now been described by way of written description and example, those of skill in the art will recognize that the invention can be practiced in a variety of embodiments, that the foregoing description and example is for purposes of illustration and not limitation of the following claims.

Claims

1. An isolated nucleic acid that encodes a CoA ligase, a non-ribosomal peptide synthesize, or a domain of an extender module of a polyketide synthase enzyme that synthesizes FK-520.

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2. The isolated nucleic acid of claim 1 that encodes an extender module, said module comprising a ketosynthase domain, an acyl transferase domain, and an acyl carrier protein domain.

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3. The isolated nucleic acid of claim 1 that encodes an open reading frame, said open reading frame comprising coding sequences for two or more extender modules, each extender module comprising a ketosynthase domain, an acyl transferase domain, and an acyl carrier protein domain.

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4. The isolated nucleic acid of claim 1 that encodes a gene cluster, said gene cluster comprising two or more open reading frames, each of said open reading frames comprising coding sequences for two or more extender modules, each of said extender modules comprising a ketosynthase domain, an acyl transferase domain, and an acyl carrier protein domain.

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5. The isolated nucleic acid of claim 2, wherein at least one of said domains is a domain of a module of a non-FK-520 polyketide synthase.

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- 6. The isolated nucleic acid of claim 1, wherein said nucleic acid is a recombinant vector capable of replication in or integration into the chromosome of a host cell.
 - 7. The isolated nucleic acid of claim 6 that is selected from the group consisting of cosmid pKOS034-120, cosmid pKOS034-124, cosmid pKOS065-M27, and cosmid pKOS065-M21.

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8. The isolated nucleic acid of claim 5, wherein said non-FK-520 polyketide synthase is rapamycin polyketide synthase. FK-506 polyketide synthase, or erythromcyin polyketide synthase.

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- 9. A method of preparing a polyketide, said method comprising transforming a host cell with a recombinant DNA vector of claim 6, and culturing said host cell under conditions such that said polyketide synthase is produced and catalyzes synthesis of said polyketide.
 - 10. The method of claim 9, wherein said host cell is a Streptomyces host cell.
- 11. The method of claim 9, wherein said polyketide is selected from the group consisting of FK-520, 13-desmethoxy-FK-520, and 13-desmethoxy-FK-506.
 - 12. A recombinant host cell that expresses a recombinant polyketide synthase selected from the group consisting of: (i) an FK-520 polyketide synthase in which at least one AT domain is replaced by an AT domain of a non-FK-520 polyketide synthase; (ii) an FK-506 polyketide synthase in which at least one AT domain is replaced by an AT domain of a non-FK-506 polyketide synthase; (iii) an FK-520 polyketide synthase in which at least one DH domain has been deleted; (iv) an FK-506 polyketide synthase in which at least one DH domain has been deleted.
- 20 13. The recombinant host cell of claim 12 that expresses an FK-520 polyketide synthase in which an AT domain of module 8 has been replaced by an AT domain that binds malonyl CoA, methylmalonyl CoA, or ethylmalonyl CoA.
- 14. The recombinant host cell of claim 12 that expresses an FK-506 polyketide synthase in which an AT domain of module 8 has been replaced by an AT domain that binds malonyl CoA, methylmalonyl CoA, or ethylmalonyl CoA.
 - 15. The recombinant host cell of claim 13, wherein a DH domain of module 5 or module 6 has been deleted.
 - 16. The recombinant host cell of claim 14, wherein a DH domain of module 5 or module 6 has been deleted.

17. A recombinant host cell that comprises recombinant genes coding for enzymes sufficient for synthesis of ethylmalonyl CoA or 2-hydroxymalonyl CoA.

18. A polyketide having the structure

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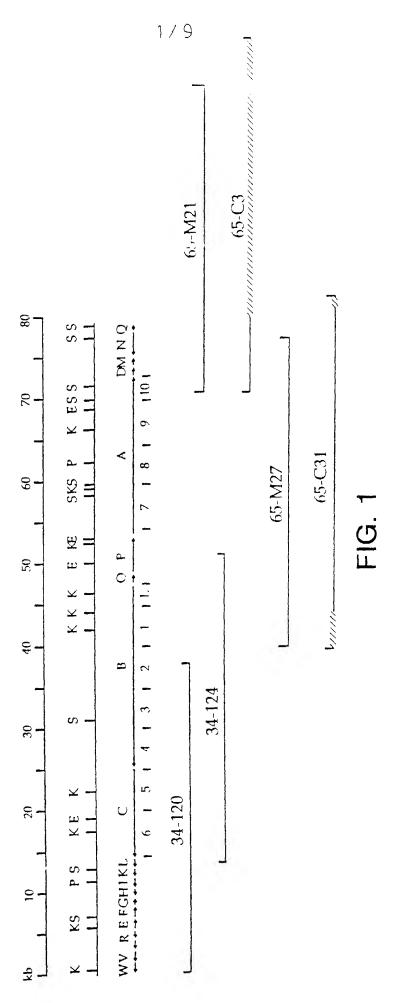
wherein, R₁ is hydrogen, methyl, ethyl, or allyl; R₂ is hydrogen or hydroxyl, provided that when R₂ is hydrogen, there is a double bond between C-20 and C-19; R₃ is hydrogen or hydroxyl; R₄ is methoxyl, hydrogen, methyl, or ethyl; and R₅ is methoxyl, hydrogen, methyl, or ethyl; but not including FK-506, FK-520, 18-hydroxy-FK-520, and 18-hydroxy-FK-506.

19. The polyketide of claim 18 that is 13-desmethoxy-FK-506.

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20. The polyketide of claim 18 that is 13-desmethoxy-18-hydroxy-FK-520.



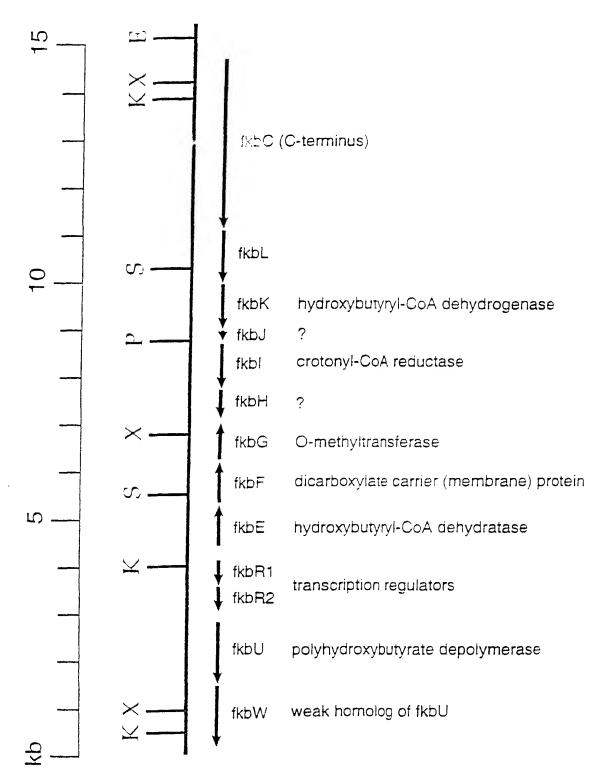


FIG. 3

FIG. 4

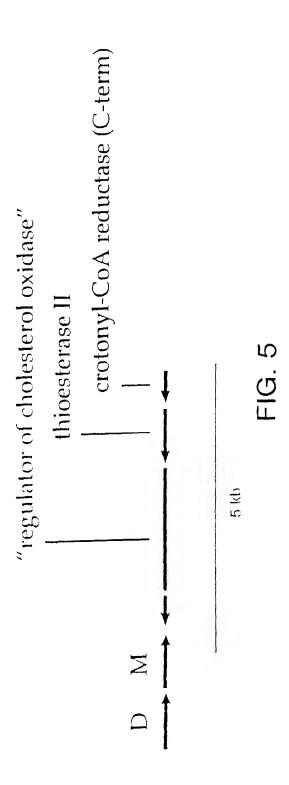
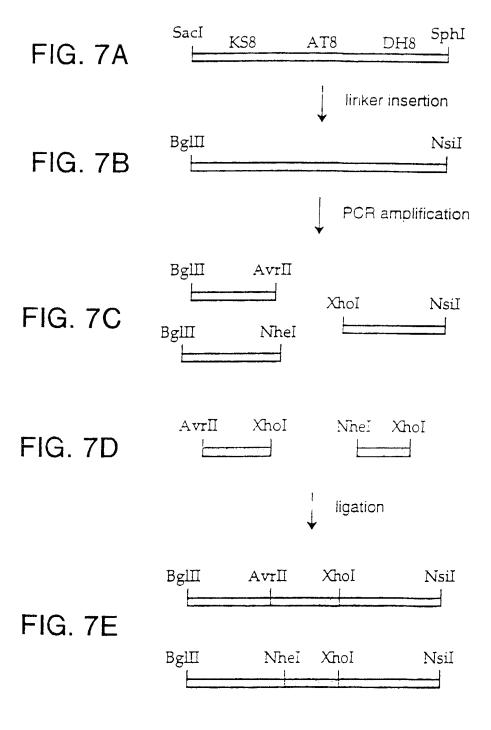


FIG. 6



INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

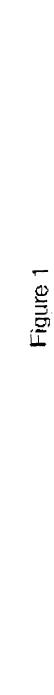
A. The indications made below relate to the microorganism r	eferred to in the description
	1-33
B. IDENTIFICATION OF DEPOSIT	Further deposits are identified on an additional sheet
Name of depositary institution American Type Cu	lture Collection
Address of depositary institution (including per and country)	אָל
10801 University Manassas, VA 221 USA	
Date of deposit	Accession Number
20 September 1999	PTA-727, PTA-728 and PTA-729
C. ADDITIONAL INDICATIONS (leave blank if not applica	ble) This information is continued on an additional sheet
D. DESIGNATED STATES FOR WHICH INDICATION All designated States	ONS ARE MADE (if the indications are not for all designated States)
E. SEPARATE FURNISHING OF INDICATIONS (lean	ve blank if not applicable)
The indications listed below will be submitted to the Internationa Number of Deposit*)	Buteau later (specify the general nature of the indications e.g., "Accession
For receiving Office use only	For International Bureau use only
This sheet was received with the international application	This sheet was received by the International Bureau on:
Authorized officer	Authorized officer
Form PCT/RO/134 (July 1992)	

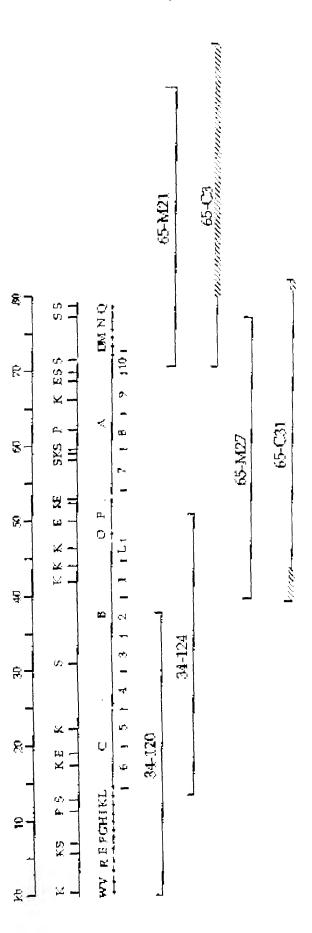
INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page 22, line 31-33						
B. IDENTIFICATION OF DEPOSIT	Further deposits are identified on an additional sheet $[X]$					
Name of depositary institution American Type Culture Address of depositary institution (including postal code and country 10801 University Blvd Manassas, VA 22110-220 USA)					
Date of deposit 20 September 1999	Accession Number PTA-726					
C. ADDITIONAL INDICATIONS (leave blank if not applicab	le) This information is continued on an additional sheet					
D. DESIGNATED STATES FOR WHICH INDICATION All designated States	NS ARE MADE (if the indications are not for all designated States)					
E. SEPARATE FURNISHING OF INDICATIONS (leave	e blank if not applicable)					
The indications listed below will be submitted to the International Number of Deposit*)	Burcau later (specify the general nature of the indications e.g., *Accession					
For receiving Office use only This sheet was received with the international application	For International Bureau use only					
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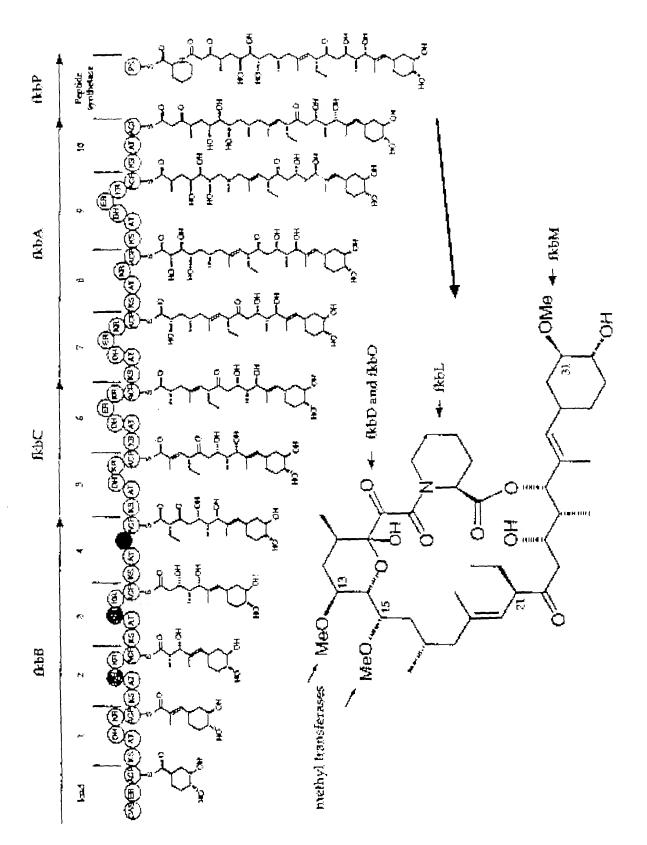


Figure 2

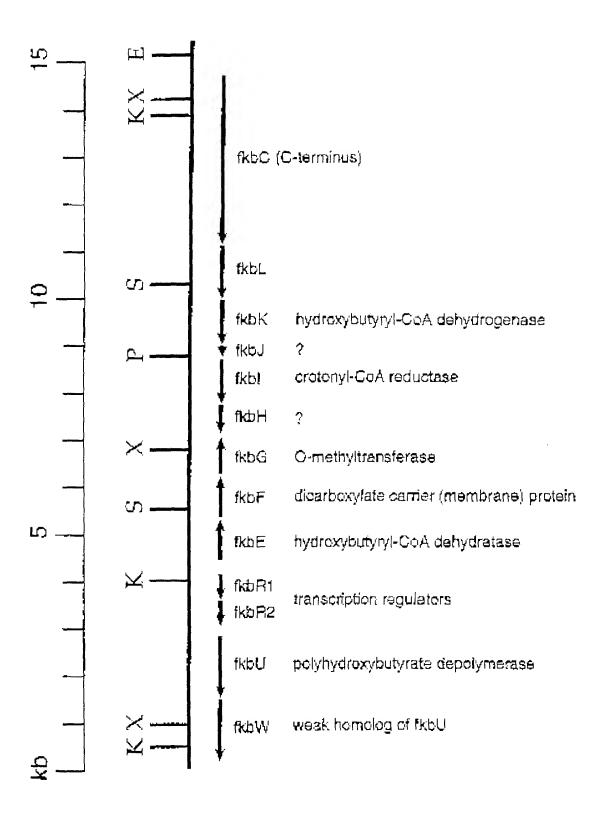
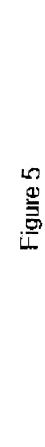


Figure 3

Figure 4



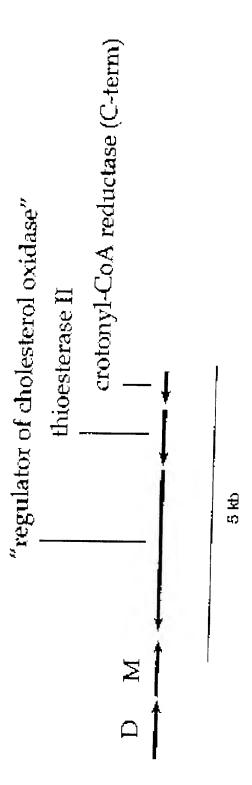


Figure 6

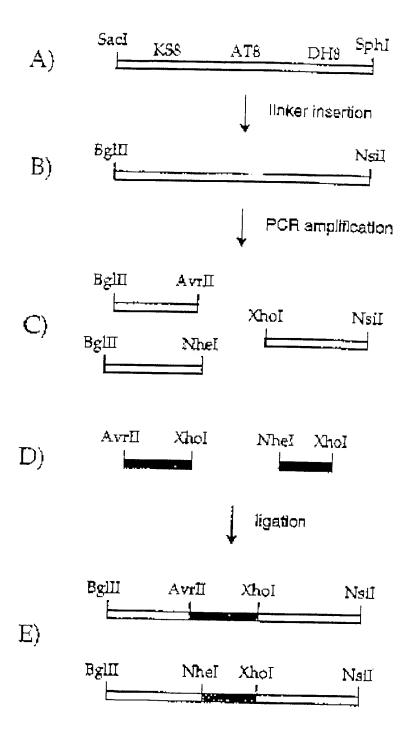


Figure 7

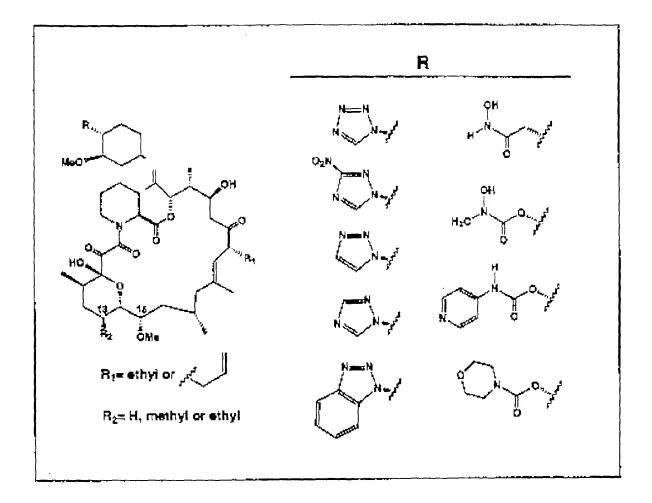


Figure 8 Part A

WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7:

C12N 15/52, 15/54, 15/62, 9/10, C12P 17/18, 19/32, C07D 498/18 // (C07D 498/18, 311:00, 273:00, 211:00)

(11) International Publication Number:

WO 00/20601

(43) International Publication Date:

13 April 2000 (13.04.00)

(21) International Application Number:

PCT/US99/22886

A3

(22) International Filing Date:

1 October 1999 (01.10.99)

(30) Priority Data:

 60/102,748
 2 October 1998 (02.10.98)
 US

 60/123,810
 11 March 1999 (11.03.99)
 US

 60/139,650
 17 June 1999 (17.06.99)
 US

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(81) Designated States: AL, AM, AU, BA, BB, BG, BR, CA, CN, CR, CU, CZ, DM, EE, GD, GE, HR, HU, IL, IS, JP, KG, KP, KR, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, US, UZ, VN, ZA, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW). Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

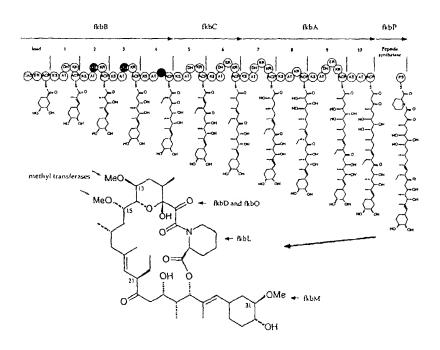
Published

With international search report.

(88) Date of publication of the international search report:

26 October 2000 (26.10.00)

(54) Title: POLYKETIDE SYNTHASE ENZYMES AND RECOMBINANT DNA CONSTRUCTS THEREFOR



(57) Abstract

Host cells comprising recombinant vectors encoding the FK-520 polyketide synthase and FK-520 modification enzymes can be used to produce the FK-520 polyketide. Recombinant DNA constructs comprising one or more FK-520 polyketide synthase domains, modules, open reading frames, and variants thereof can be used to produce recombinant polyketide synthases and a variety of different polyketides with application as pharmaceutical and veterinary products.

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A. CLASSIFICATION OF SUBJECT MATTER IPC 7 C12N15/52 C12N15/54 C12N15/62 C12N9/10 C12P17/18 C12P19/32 C07D498/18 //(C07D498/18.311:00.273:00.211:00)

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B. FIELDS SEARCHED

Minimum gocumentation searched "classification system followed by classification symbols: IPC 7 C12N C12P C07D

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WPI Data, PAJ, MEDLINE, STRAND, BIOSIS, EMBASE, CHEM ABS Data

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Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Van de Kamp, M				

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